THE PROTECTIVE EFFECTS OF ESTRADIOL ON SPORADIC AND INFLAMMATION-ASSOCIATED COLON CANCER

A Dissertation

by

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ABSTRACT

Epidemiological studies suggest pre-menopausal women have a reduced risk for sporadic and inflammation-associated colon cancer compared to post-menopausal women and men. The studies presented herein aim to determine the protective mechanisms of estradiol (E_2) during sporadic and inflammation-associated colonic carcinogenesis.

When investigating the role of E_2 and fish oil at the earliest stage of sporadic colon cancer development, E_2 had no effect on DNA adduct formation while dietary fish oil significantly reduced DNA adduct formation. Contrarily, E_2 significantly induced apoptosis of damaged colonocytes while fish oil was not protective.

In an *in vivo* model of inflammation-associated colon carcinogenesis with E_2 administered following induction of DNA damage and initiation of inflammation, E_2 treatment was associated with decreased colon tumor size and number in wild type (WT) but not estrogen receptor (ER) β knockout (ERβKO) mice. Interestingly, apoptosis was reduced and proliferation increased by E_2 in these tumors in WT mice. This may be due to the altered ER expression in these tissues as the tumors developed, with ERβ expression decreasing concomitantly with ERα expression increasing.

Contrary to the protective effect of E_2 on inflammation-associated colon tumor formation, which was dependent on ERβ, during acute inflammation in the colon E_2 was protective against inflammation in both WT and ERβKO mice and injury in ERβKO mice. The protection against inflammation is likely due to the reduction of pro-

inflammatory cytokine expression by E_2. Apoptosis and proliferation were decreased and increased in the proximal and distal colon respectively in ERβKO mice.

In vitro studies further elucidated the roles of ERα and ERβ in colonocytes. E_2 and ERβ, but not ERα, specific agonists reduced cell number and induce apoptosis in nonmalignant colonocytes. This effect was lost in the presence of mutated p53. In ERα overexpressed nonmalignant colonocytes, E_2 had no effect on cell number while ERβ agonist and ERα agonists decreased and increased cell number respectively.

These studies suggest that E_2 is protective in the colon and ERβ is required for protection against carcinogenesis but not protection against inflammation. Additionally, the protection against colon carcinogenesis is likely p53 mediated.

DEDICATION

I would like to dedicate this body of work to my family and friends for being with me throughout the years. Without them I wouldn't be where I am today.

For my father: thank you for instilling a love for science in me at a young age. I will never forget all the time you spent helping out in my elementary school science class, playing with liquid nitrogen in the back yard and making soda from scratch. And how many kids can say they had a homemade hovercraft in the garage?

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INTRODUCTION AND LITERATURE REVIEW

Colon Cancer Statistics, Pathogenesis and Risk Factors

In the United States, colon cancer is the third leading cause of cancer related deaths in men and women, falling behind lung cancer and prostate or breast cancer in men and women respectively [1]. Fortunately, however, in recent years the number of colon cancer cases has actually decreased [2]. This is due, in part, to improved knowledge of preventative measures and better methods of early detection. Despite this, the fact remains that over 50,000 people are expected to die from colon cancer in the United States in 2013.

Development of colon tumors is a time intensive process, oftentimes requiring anywhere from 10 to 15 years for tumors to form [3]. Colon cancer pathogenesis occurs in three primary physiological steps: initiation, promotion, and progression. Initiation is characterized by damage to the DNA resulting in the dysregulation of normal gene functions. These mutations can be the result of several factors including genetics, lifestyle, and diet. An indicator of impending DNA damage that occurs during colon carcinogenesis but is primarily measured in experimental models is DNA adducts. DNA adducts are formed when carcinogens covalently bind to DNA. With the carcinogen bound, DNA replication cannot proceed normally and errors are incorporated into the daughter strands unless DNA repair mechanisms correct the error.

One gene often affected during the initiation phase of colon carcinogenesis is the adenomatous polyposis coli gene (APC) [4]. Dysregulation of APC in turn is known to cause over expression of mutant β-catenin and can lead to mutations in the proto-oncogene k-*ras* [5, 6]. The physiological results of the mutations to these genes are decreased apoptosis and increased proliferation of the affected cells [5-7]. In healthy colon tissue, proliferation is confined to the base of colonic crypts with cells migrating up towards the lumen as they differentiate and eventually sloughing off; a process which takes between 4-6 days [8]. As cells lose the ability to properly regulate proliferation, the proliferative zone expands and S-phase cells can be found throughout the entire height of the crypt [9].

The second phase of colon cancer development, promotion, is hallmarked by the formation of polyps. Adenomas, one classification of polyp, are most likely to become cancerous. Adenomas are relatively common and it is estimated that between one third to one half of the population will develop at least one adenoma within their lifetime [10, 11]. Fortunately, less than 10% of adenomas develop into adenocarcinomas [12]. Further mutations to oncogenes and tumor suppressor genes accumulate in affected cells, in part because the first mutation makes it easier for subsequent mutations to occur. Some of the most commonly altered genes in colon tumors include APC and k-*ras*, as previously mentioned, as well as *p53,* deleted in colorectal carcinoma *(DCC)*, and Dpc4 [7, 13-15].

Ultimately, adenocarcinomas can infiltrate through the lining of the colon, invade into neighboring tissues, and cancer cells can metastasize to distant locations. Each of

these occurrences is part of the third phase of colon carcinogenesis, progression. Here, cancerous cell growth outpaces healthy cell growth and the tumor has acquired several mutations leading to increased angiogenesis, dysregulated proliferation and resistance to apoptosis.

Clinically, there are five categories of colon cancer development, ranging from 0 to 4. Stage 0 is alternatively referred to as carcinoma in situ. At this stage, the developing tumor remains contained within the lining of the colon. In Stage 1, the tumor has penetrated through several layers of the colon, but has not spread beyond the muscle layer. Once the tumor has breached the muscle layer but has not infiltrated into the lymph system it has developed into a Stage 2 malignancy. Stage 3 has entered the lymph nodes but not metastasized to distant organs, which happens in Stage 4. Stages 0 and 1 are often treated by simple removal of the affected tissue. For Stages 2 and 3, surgical removal of the tumor is often accompanied with chemotherapy. Once Stage 4 colon cancer has been reached, surgical colon tumor removal is aimed more at relieving symptoms or preventing further complications associated with the disease as depending on the extent of the metastasis, it may be impossible to fully extract the cancerous tissue. Here again, chemotherapy or radiation therapy may be used, as a method to relieve the patients symptoms.

There are several risk factors for developing colon cancer, among them being age with persons over 50 years being at a significantly higher risk. Genetics can also play a large role. The two most common genetic disorders resulting in colon tumor development are familial adenomatous polyposis (FAP) and Lynch Syndrome, together

accounting for between 4-6% of all colon cancers [16, 17]. FAP is caused by inherited mutations to APC resulting in the formation of hundreds of polyps and persons with this disease often develop cancer by the time they are 20 years of age. Most persons with FAP undergo colonectomies to treat their condition. Persons with Lynch Syndrome do not develop near as many polyps however they have an 80% overall lifetime risk for developing colon tumors. This condition is due to mutations in several different genes that regulate DNA repair.

In addition to age and genetics, lifestyle and diet are large contributing factors for colon carcinogenesis. Studies have shown that persons with higher BMIs and sedentary habits are at a significantly increased risk for colon tumor formation [18]. In regards to diet, several components have been demonstrated to both increase and decrease colon cancer risk depending on quantity and frequency of consumption. Among the foods known to increase cancer risk are: processed meat, red meat, foods high in saturated fatty acids, highly refined grains and starches, excessive alcohol and added sugar [19-22]. Proposed mechanisms behind the increased risk include altering the membrane of epithelial colonocytes and increasing insulin and insulin-like growth factor-1, each of which can result in increased proliferation in cells [20, 23-25]. On the other end, consumption of whole grains, fruits and vegetables, dietary fiber, tea, soy and fish rich in n-3 polyunsaturated fatty acids are all tied to a reduced risk [26-33]. In general, these beneficial compounds act as chemopreventatives and protect the tissue from developing tumors.

Screening for colon cancer is advised once a person reaches 50 years via a colonoscopy with subsequent colonoscopies every ten years. Persons who have family history of colon cancer or who are otherwise known to be at an increased risk are suggested to be screened earlier and more frequently [34]. Detection of colon cancer is relatively easy with regular screening, and when caught and treated early enough, the five year survival rate is around 90%. Unfortunately, only four out of every ten people with colon cancer have their cancer detected at this early stage due to the fact that they are not getting themselves tested for the disease [1]. This is due, in part, to limited accessibility and affordability of the procedure.

The financial burden of having and treating colon cancer has been steadily increasing over the past few decades. By 2020, it is predicted that medical care expenditures in the United States for colon cancer will increase over 40% to $14.02 billion [35]. Costs for each individual inpatient rose 37.6% from 1998 to 2007 [36]. Fortunately, it is suggested that if people were to make moderate changes to their diet and lifestyle, reducing habits that promote cancer growth and increasing activity and consumption of beneficial dietary components, then up to 70% of the current costs associated with colon cancer could be prevented [37, 38]. For colon cancer, chemopreventative strategies are not only fiscally beneficial but could lead to the prevention of colon cancer related deaths in numerous people.

Fish Oil and Colon Cancer

Epidemiological studies show that populations with diets high in fatty fish, and subsequently fish oil, are at a reduced risk for developing colon cancer [39]. Fish oil is available as fish oil capsules or directly from consumption of fatty fish. Fatty fish and fish oil capsules contain the n-3 polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). DHA is a 22 carbon fatty acid with six double bonds while EPA has 20 carbons and five double bonds (Figure 1.1). Both of these n-3 fatty acids can also be synthesized from the essential fatty acid, α-linolenic acid, through elongation and desaturation of the carbon-carbon bonds. This conversion, however, is much less efficient than absorbing the compounds directly from the diet. Fish high in DHA and EPA content include salmon, tuna, and swordfish. Farm raised fish have low levels of the n-3 PUFAs compared to wild caught fish due to the fact that the fatty acids are not produced by the fish themselves but rather are obtained through the fish's diet of algae.

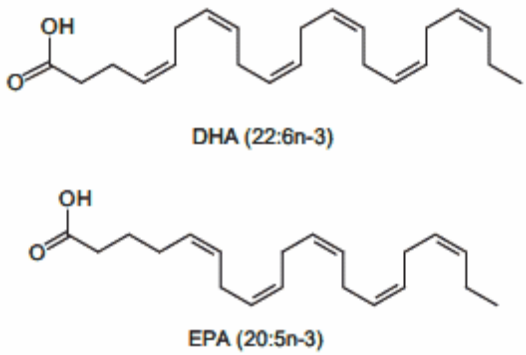

Figure 1.1. Structures of the n-3 PUFAs, DHA and EPA.

There are several proposed mechanisms by which fish oil is suggested to prevent colon cancer. DHA has been shown to initiate cell cycle arrest and induce apoptosis [40]. In LS-174T cells, a human colon tumor cell line, treatment with DHA reduced proliferation and suppressed prostaglandin E2 (PGE2) and cyclooxygenase-2 (Cox-2) induced by arachidonic acid [41]. Aberrant Cox-2 expression is an indicator of colon tumor invasion and metastasis and in turn leads to overexpression of PGE2 which also promotes cancer cell growth [42]. In addition to affecting Cox-2 and PGE2, DHA is known to upregulate the phosphatidylinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways, both of which are often involved in inducing apoptosis [43].

Epidemiological studies investigating EPA have found that in persons with FAP, EPA consumption significantly reduced the size and number of polyps [44]. For this PUFA, studies suggest that suppression of angiogenesis may be a mechanism of action [45]. Other studies have found that dietary EPA in APCmin/+ mice significantly reduced intestinal cell proliferation and suppressed Cox-2 expression and inhibited nuclear translocation of β-catenin [46].

As a whole, dietary fish oil has been demonstrated *in vivo* to reduce the formation of azoxymethane (AOM) induced DNA adducts [47-49]. Combinatory fish oil and pectin (a soluble fiber) diets in mice have repeatedly proved successful at preventing early markers of colon carcinogenesis as well as colon tumor formation itself [47-51]. Induction of apoptosis is consistently demonstrated as a mechanism of action via modulation of several pro and anti-apoptotic proteins including B-cell lymphoma 2

(Bcl-2), peroxisome proliferator-activated receptor delta (PPARdelta), PGE2 and prostaglandin E3 (PGE3) [52, 53].

Estrogen and Colon Cancer

Estrogens are the primary sex hormones in females. Biosynthesis of estrogens in women principally occurs in the ovaries or, during pregnancy, is produced by the placenta. Aromatase activity in adipocytes can also convert testosterone into estrogen. De novo synthesis of estrogen begins with cholesterol which through a series of reactions and multiple interconnected pathways is converted into one of the three kinds of estrogen [54].

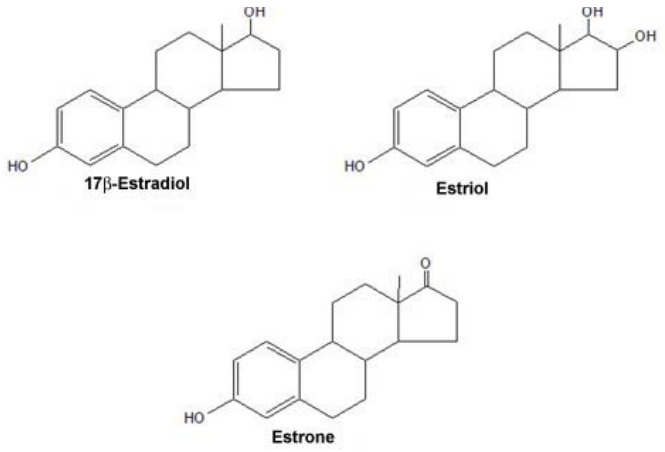

Figure 1.2. Skeletal structures of the three natural forms of estrogen: 17β-estradiol, estriol, and estrone.

The three naturally occurring forms in most animals are: estrone, estriol, and 17β-estradiol (E_2) with E_2 being the predominant active form (Figure 1.2). Estrogens are required for the development of secondary sex characteristics in women and are an

integral part of the female reproductive system [55]. Additionally, estrogens have been demonstrated to have important functions in bone, brain, adipose, and cardiovascular tissues as well as in the male reproductive system [56, 57].

Post-menopausal women receiving hormone replacement therapy (HRT) or estrogen replacement therapy (ERT) are at a reduced risk for the disease compared to post-menopausal women not on HRT or ERT [58-62]. The protection observed in the post-menopausal women was lost however, if they discontinued using the therapies. *In vivo* studies investigating the function of estrogens during colon carcinogenesis have demonstrated that treatment with E_2 and metabolic precursors to E_2 reduces the formation of colon tumors and of aberrant crypt foci (ACF), which are early markers of colon tumorgenesis [63-65]. These data suggest that estrogen may play a role in preventing colon cancer.

The evidence for E_2 being a true chemopreventative compound and not an effective treatment for pre-existing colon tumors comes primarily from *in vitro* experiments using colon tumor cell lines. While there is some conflicting data, in most cases in malignantly transformed cell lines E_2 has no physiological effect [66-68]. Contrarily, in Young Adult Mouse Colonocytes (YAMC), a non-malignant colon cell line, E_2 treatment results in a decrease in cell number [63]. These data point to the idea that the window for opportunity for effective treatment with E_2 in colon cancer is prior to tumor formation.

The mechanisms behind this observed protection both *in vivo* and *in vitro* is proposed to be through the modulation of apoptosis. *In vitro*, E_2 treatment in YAMCs

has been shown to upregulate apoptosis, in part through a p53 mediated pathway [69]. p53 is an important tumor suppressor gene that is found to be altered in over 50% of all cancers due to mutations that are thought to occur later during colon tumorgenesis. In a normally functioning cell, p53 expression is induced in response to DNA damage or other forms of cellular stress. Subsequently, p53 initiates cell cycle arrest and apoptosis. This protects the cell from accumulating multiple mutations and becoming cancerous. Direct mutation of p53 is not the only way that p53 can be dysregulated; oncogenic transformation of other proteins within the p53 pathway, such as murine double minute 2 (Mdm2) [70]. Dysregulated Mdm2 results in rapid degradation of p53, reducing the cell's ability to initiate apoptosis. Weige et al. demonstrated that while E_2 reduced cell number and induced apoptosis in YAMCs, this effect was lost in YAMC mp53/neo cells, a homologous cell line containing a $p53^{175H}$ mutant gene [69]. This demonstrates that functional p53 is required for the protective effect of E_2.

Other *in vitro* studies have found that E_2 suppresses activation of protein kinase B (AKT) and extracellular-signal-regulated kinase 1/2 (ERK1/2) resulting in suppression of PGE2 and Cox-2 [71]. AKT is a protein whose overexpression often occurs early on during colon carcinogenesis. This overexpression causes inactivation of Mdm2 which in turn decreases p53 resulting in reduced apoptosis of cells. ERK1/2 on the other hand initiates cell cycle progression and proliferation. Overexpression of either protein results in uncontrolled growth of cells and promotes tumor formation.

In vivo experiments corroborate *in vitro* data. Apoptosis has been demonstrated to be upregulated by E_2 at the ACF stage in an AOM induced model of colon

carcinogenesis [63]. A separate study investigating the effect of E_2 on ACF formation in mice heterozygous for p53 observed that the protective effect of E_2 was partially lost compared to wild type (WT) mice, further demonstrating the interplay between E_2 and p53 (Weige, data not published). Interestingly, apoptosis has also been observed to be upregulated in healthy animals not subjected to carcinogen [72, 73]. It is important to note that the increase in apoptosis in these animals was minimal (less than 0.1%) and was not visibly disruptive of healthy tissue physiology. This fact again supports the chemoprotective role of E_2 in the colon.

Estrogen Receptors

The effects of E_2 in the body are instigated by the binding of ligands to the nuclear receptors known as estrogen receptors (ER). There are two primary subtypes of ER, ERα and ERβ, which in general have physiologically opposite effects on tissues and systems. ERα is known to initiate proliferation and is associated with increased cancer growth in the tissues where it is the predominate ER, such as in the breast [74, 75]. ERβ on the other hand is associated with decreased growth, increased apoptosis and cell cycle arrest. These ERs are expressed throughout the body in numerous tissues. ER expression of one subtype or the other is not mutually exclusive and both receptors can be present in the same tissue, despite their opposite actions [76].

Activation of the ERs elicits downstream effects in one of several ways. The primary method starts when a ligand binds to the ER on the nuclear membrane. The receptors then dimerize and translocate into the nucleus where they bind to estrogen

response elements (ERE) found in the promoter region of target genes. Once bound, activation of the ERE causes the recruitment of additional proteins to the promoter region that will either result in the increase (co-activators) or decrease (co-repressors) expression of the target gene mRNA and protein culminating in a physiological response.

A secondary method by which ERs regulate transcription is called transcriptional cross talk and does not involve direct binding to DNA. Instead, this mechanism relies on the interaction of ERs at activator protein-1 (AP-1) binding sites with Fos and Jun proteins. The ER-AP-1 complex binds p160 co-activators via ER activation function domains on the ER and together this stabilizes protein complexes containing c-Jun and transcriptional co-activators at the promoter of the target genes [77]. Some genes that are regulated using this mechanism include cyclin D1 and insulin-like growth factor 1 [78, 79]. ERs can also activate genes that have promoter regions rich in GC content in a similar manner by interacting with the Sp1 transcription factor [77].

In addition to genomic actions of ERs, there is evidence that ER activation at the plasma membrane, as opposed to the nuclear membrane, can elicit more rabid responses in cells not relying on RNA and protein production. Here, it is suggested that ERs congregate in caveolae of endothelial cells and upon ligand binding can activate endothelial nitric oxide synthase through protein kinase phosphorylation [80]. Alternatively, ligand-bound plasma membrane ERs may also activate tyrosine kinase receptors including IGF-1, epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (HER2) [81-83].

ERα is the predominant ER in reproductive tissues including breast, endometrial, ovarian and hypothalamic tissue. It is encoded for on chromosome 6 in the human genome. Conversely, ERβ is located primarily in heart, endothelial, bone, kidney, lungs, brain, and intestinal cells and is encoded for on chromosome 14. Both ERs are composed of three principal domains: the N-terminus domain, the DNA-binding domain and the ligand-binding domain. Activation function (AF) domains are located within the N-terminus domain and the ligand-binding domain. AF domains are requisite for regulation of the transcription activities of the ERs and are sites for cofactor binding and dimerization upon activation of the ER. Between ERα and ERβ, the DNA-binding domain has the highest homology at over 95%. The ligand-binding domain shares around 55% homology and the N-terminus domain only has about 15% homology between the ER subtypes. In addition to the poor sequence homology of the N-terminus domain, it is also significantly shorter in ERβ compared to ERα (Figure 1.3) [84]. The physiologically different effects between ERα and ERβ are thought to arise from the disparate amino acid sequences comprising the AF2 domain within the ligand binding domain.

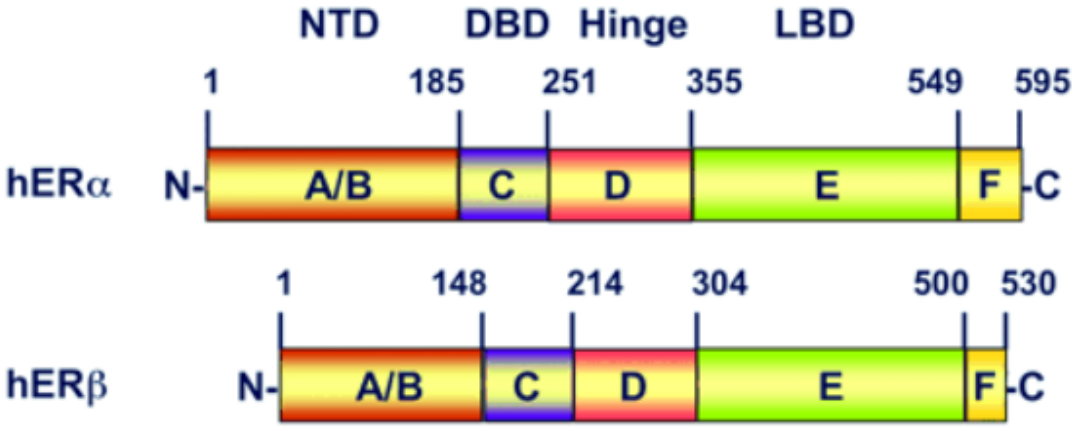

Figure 1.3. Comparative diagram of the differences in structural domains between ERα and ERβ. Adapted from Koehler et al. [85].

In the colon, ERβ is the primary ER and scientific evidence points to this ER being the receptor through which E_2 exerts its protective effect. Oral estrone was equally effective at reducing AOM induced tumor multiplicity in ERα WT and ERα knockout (KO) mice, ruling out ERα as the mediator of estrogenic effects in the colon [64]. In experiments conducted in ERβKO, however, E_2 treatment had no effect on ACF formation or on induction of apoptosis. In contrast, WT mice from the same study had reduced ACF formation and an induction of apoptosis in the presence of E_2 [63]. Additionally, treatment with ERβ specific agonists in ovariectomized rats also induced apoptosis of colonocytes [86].

Both epidemiological studies and experimental models have demonstrated that as colon tumors develop, the expression of ERβ in colonocytes decreases [87, 88]. This loss of ERβ may be the reason that malignantly transformed cells are no longer

responsive to treatment with E_2. In humans, it was observed that colon tumors not expressing ERβ were more likely to be at a higher stage of cancer and that persons with these tumors were at a higher risk for death compared to those with colon tumors expressing ERβ [89]. These data highlight the importance of ERβ during colon carcinogenesis.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a collection of diseases affecting the small intestine and the colon. The two main conditions associated with IBD are Crohn's Disease and ulcerative colitis. Common symptoms of IBD include fever, diarrhea, rectal bleeding, and weight loss [90]. These symptoms come and go at varying levels of severity. In the United States, over one million people are currently diagnosed with IBD. Most commonly, IBD occurs in persons in their twenties and thirties with a second peak of flare-ups in the same persons later in life. Both forms of IBD occur due to aberrant immunological responses to antigens in normally occurring proteins in genetically susceptible hosts. This results in disruption of intestinal membrane function, creating large gaps between epithelial cells that bacteria can pass through, which in turn leads to further immunological responses all leading to inflammation of the intestine.

Both genetic and environmental factors can lead to an increased risk for developing IBD. Epidemiological studies suggest that having a first-degree relative with IBD are up to 20 times more likely to have IBD themselves compared to those without affected relatives. While Mendelian genetics have not been identified to cause IBD,

studies have found conserved regions on chromosomes 3, 5, 7, 18, 19 and the X chromosome that are linked with both Crohn's disease and ulcerative colitis and additional regions on chromosome 16 and 12 that are specific to Crohn's disease and ulcerative colitis respectively [90]. Multiple genes that have been linked with IBD, among them are tumor necrosis factor (TNF) and interleukin-10 (IL-10). Environmental factors that have been tied to development of IBD include the diet, the microflora composition of the intestines and habitual use of non-steroidal anti-inflammatory drugs [91]. Interestingly, smoking has been observed to protect against ulcerative colitis but increases the risk for Crohn's disease [92, 93].

Crohn's disease occurs mostly in the colon and the ileum but can occur anywhere along the gastrointestinal tract. Symptoms vary depending upon the location of the flare-up; if in the esophagus patients may present with dysphagia, heartburn or chest pain [94]. Alternatively, gastroduodenal Crohn's disease causes weight loss, upper abdominal pain, nausea and vomiting [95]. The disease is characterized by a cobblestone-like appearance of the affected areas; distribution is segmented. Fistulas (abnormal connections between body cavities) and fissures (cuts or tears) are fairly common and the ulcers are deep, extending into the submucosa. The inflammation in Crohn's disease is associated with neutrophil infiltration into the epithelial cells and into the lumen of colonic crypts. This leads to the formation of crypt abscesses and eventual destruction of the crypt structure and atrophy of the intestine [96].

Conversely, in ulcerative colitis, the distribution of affected tissue is continuous and contained exclusively in the colon and rectum. Ulcers are superficial and fistulas

and fissures are absent. The inflammation in ulcerative colitis is confined to the submucosa and mucosa in contrast to Crohn's disease where the inflammation affects the entire wall of the intestine. As with Crohn's disease, neutrophils infiltrate into crypts creating abscesses in the affected tissue. There is reduced production of mucins from goblet cells and an aberrant Paneth cell distribution associated with the disease [96]. The colon may be shortened in patients with ulcerative colitis and have thicker intestinal walls. Crypt structure is also altered in ulcerative colitis: crypts are shallower and do not extend to the muscle membrane layer [97].

Currently, there is no cure for IBD and treatment can only alleviate the symptoms to allow the person a more normal life. The main prescribed treatment options are the administration of anti-inflammatory drugs and immune-suppressants such as corticosteroids and anti-TNFα reagents. Other viable treatments include probiotics and prebiotics with the aim to modulate the intestinal microflora to prevent flare-ups of the disease [98-100] .

Inflammation-associated Colon Cancer

Having IBD increases a person's risk for developing colon cancer. Ulcerative colitis has the larger effect, increasing cancer risk by as much as 20% while Crohn's disease increases the risk for developing colon tumors by up to 8% [101, 102]. The tumors that result from inflammation-associated colon cancer are distinct from those that develop in sporadic colon cancer. These tumors often occur in multiples and are more

flat and infiltrating. In addition, inflammation-associated colon tumors are more likely to be of a higher grade and mucinous.

There are several methods by which the inflammation associated with IBD is proposed to promote the development of colon cancer. In sporadic colon cancer, aberrant activation of the Wnt/β-catenin signaling pathway is an early occurrence in over 90% of cases. This activation causes proliferation of cells and prevents older cells from apoptosing and sloughing off into the lumen [103, 104]. In the presence of inflammation, it has been suggested that β-catenin activity is upregulated even in the absence of genetic mutations to regulatory genes [105]. β-catenin nuclear accumulation can be caused by several inflammatory pathways, including NF-κB, PI-3K, and Akt [104]. The upregulation of β-catenin via these inflammatory pathways could be a way in which inflammation-associated colon cancer can bypass the need for an initiating genetic mutation. Regardless of the lack of initial mutation, the upregulated proliferation caused by the inflammation itself increases the risk for developing future mutations.

Inflammation can also affect cancer progression and metastasis. Inflammation facilitates epithelial-mesenchymal transition (EMT), which is required for metastasis, through the production of cytokines. TNFα, IL-1 and IL-6 have been observed *in vitro* to induce metastatic behavior in malignantly transformed colon cell lines [106-108].

Another important player in the development of inflammation-associated colon cancer is the gut microflora. Colon tumor formation in gnotobiotic mice induced by a combination of dextran sulfate sodium (DSS) (an inducer of intestinal colitis) and AOM was found to be reduced compared to mice with normal gut microflora [109]. The

microbes in the intestines induce the expression of pro-inflammatory cytokines such as interferon-γ (IFNγ), IL-10 and IL-17 from immune cells in the gut which in turn can promote proliferation of cells [104]. Bacteria may be beneficial for the prevention of inflammation-associated colon cancer: treatment with probiotics in rats has been demonstrated to inhibit IBD and delay the formation of dysplasia in the colon [110].

Estrogen and Inflammation-associated Colon Cancer and IBD

As with sporadic colon cancer, E_2 is also associated with a decreased risk for developing inflammation-associated colon cancer. Söderlund et al. observed that men with IBD were 60% more likely to develop colon tumors than women with IBD [111]. In regards to IBD itself, studies observing the occurrence of IBD symptoms in pre-menopausal women have found that patients with Crohn's disease, but not ulcerative colitis, displayed a worsening of their IBD symptoms during menses [112]. This is the point during the menstrual cycle that E_2 concentrations are lowest in the body and suggests that during the rest of the women's cycles, the higher E_2 concentrations could be protecting them from their IBD.

Animal studies investigating E_2's effect on inflammation and inflammation-associated colon tumor development have returned conflicting results, however. Saleiro et al. induced inflammation-associated colon cancer in WT and ERβKO female mice using DSS and AOM. In these mice, loss of ERβ was associated with an increased number and size of polyps as well as a higher grade of dysplasia and inflammatory score [113]. Although E_2 concentrations were the same in both genotypes in this particular

study, it is important to bear in mind that ERβ is the primary ER in the colon and loss of the ER would negate most effects of E_2 in the affected tissue. The fact that in this case the WT mice were protected demonstrates the efficacy of E_2 for preventing inflammation-associated colon cancer. In this same study, WT mice had reduced expression of β-catenin, NF-κB, inducible nitric oxide synthase (iNOS), and several pro-inflammatory cytokines including TNFα, IL-6, IL-17, and IFNγ further demonstrating the protective effect of E_2 through activating ERβ [113].

A separate study also using the DSS/AOM model showed that E_2 actually promoted inflammation-associated colon tumor formation. That study used intact, ovariectomized (OVX), and OVX mice receiving E_2 and/or progesterone. E_2 treated mice in this case had more polyps, larger polyps, and increased colonocyte proliferation compared to control. Additionally, when they conducted the same study design in ERαKO and ERβKO mice they found that loss of either ER was protective against the disease, suggesting that both ERs play a role in inflammation-associated colon tumorgenesis [114]. Similar to Saleiro et al., the authors conducted an experiment using intact female mice. In contrast to the observations of Saleiro et al, Heijmans et al. found that intact WT mice had an increase in the formation of polyps. The disparity in the outcomes observed between these two studies is likely due to differences in the AOM/DSS models used; while both groups injected the mice once with 10mg/kg AOM, Saleiro et al. administered a single 2.5% round of DSS whereas Heijmans et al. used three rounds of 1.5% DSS [113, 114].

Other animal studies have looked at the effect of E_2 and other estrogenic compounds on colonic inflammation alone without associated tumor formation. In DSS induced colitis, E_2 treatment is correlated with a worsening of inflammation. Heijmans et al. observed that E_2 in OVX mice increased the disease activity index, IL-6 secretion, and proliferation of colon cells [114]. Similarly, Verdú et al found that compared to control, E_2 treated mice had vastly higher disease severity and histological scores [115].

Interestingly, however, other models of inducing inflammation have seen a protective effect of E_2. A reduction of inflammatory score was observed with E_2 in dinitrobenzene sulfonic acid (DNB) colitis accompanied by reduced myeloperoxidase (MPO) activity suggesting diminished infiltration of polymorphonuclear leukocytes (PMN) [115]. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) induced colitis has also been suppressed by forms of E_2: estradiol benzoate reduced expression of macrophage migration inhibitory factor and IL-1β, both of which are upregulated during inflammation, compared to control [116]. Phytoestrogens, which are plant derived compounds that have estrogenic action and are known to bind ERs, have also been shown to modulate TNBS induced colitis: dietary genistein in rats reduced the expression of Cox-2 and MPO [117].

The differential responses to E_2 in the different models of inflammation are probably due to the mechanisms by which each chemical induces the inflammation in the animals.

Methods of Inducing Inflammation

There are several ways to induce experimental colitis. Primary among them are the chemicals DSS and TNBS. Each causes inflammation using distinct mechanisms resulting in colitis that mimics Crohn's disease (TNBS) and ulcerative colitis (DSS).

TNBS is administered intrarectally dissolved in ethanol. The ethanol is proposed to increase the permeability of the intestinal lining allowing the TNBS to infiltrate into the sub-epithelial spaces of the tissue. Once inside the tissue, the TNBS haptenates endogenous colon proteins as well as microbial proteins. These haptenated molecules then elicit an allergic response from the host's immune system. T helper (TH) 1 and TH17 cells express the pro-inflammatory cytokines IL-2, IL-12, IL-18, IFN-γ and TNF-α and are responsible for the subsequent inflammation [118, 119]. Individual protocols for inducing TNBS colitis vary widely depending on the genetic background of the animal being used and by the experimental outcomes being measured. Dosages of TNBS range from 0.5 mg to 6 mg per mouse, the ethanol it is dissolved in from 35% to 50% and the number of administrations range between one and three. For acute colitis studies, only one dose of TNBS is used and the mice are usually sacrificed between 3 and 5 days afterwards. Chronic colitis is induced using 2 to 3 rounds of TNBS with sacrifice occurring between days and several weeks following the last treatment [119-121].

DSS is administered to the animals via the drinking water. Here, the mechanism is believed to be a direct cytotoxic effect on intestinal epithelial cells of crypts causing a loss of integrity in the mucosal barrier. Bacteria are then able to penetrate into the sub-

epithelial space where they stimulate immune cells to mount a response [121]. Due to the fact that T and B cell deficient mice still developed colitis in response to DSS, it is believed that the innate immune system and not the adaptive immune system plays the larger role in DSS colitis [122]. Animals often present with weight loss, bloody diarrhea, ulcerations, and infiltration of granulocytes [121]. As with TNBS, there are models for both acute and chronic colitis using DSS. Again, acute colitis involves a single round of treatment with sacrifice within days and chronic colitis is generally induced using three rounds of DSS separated by 14 days on regular water [120].

There are other models of experimental colitis; however, they are not widely used. Among them are mutant strains of mice, C3H/HeBir and SAMP1/Yit, genetic KO and transgenic mice, and administering $CD4^+$ T cells in immunodeficient mice [123-127]. These models, however, are not as widely used as DSS and TNBS and are not as well characterized.

CHAPTER II

DIETARY FISH OIL REDUCES DNA ADDUCT FORMATION WHILE ESTRADIOL UPREGULATES APOPTOSIS IN RESPONSE TO DNA DAMAGE IN THE RAT COLON*

Introduction

Colon cancer is the third leading cause of cancer related deaths in both men and women. Overall, the disease is the second highest cause of cancer related deaths [1]. Epidemiological as well as experimental data demonstrates that colon cancer can be affected by many lifestyle choices including the diet [128-131]. Fish oil (FO), which contains high levels of *n*-3 polyunsaturated fatty acids (PUFA) has been shown to reduce the incidence of colon cancer [32, 132, 133]. One way in which *n*-3 PUFA can be protective against colonic malignancy is by up-regulating apoptosis in colonocytes [43, 134, 135]. Pathways involved with apoptosis are often dysregulated in cancer development resulting in reduced apoptosis of cells which have sustained DNA damage. In turn, this results in an increased risk for tumor formation [136, 137]. *In-vitro* studies have shown that treatment of colon cancer cells with docosahexaenoic acid, a *n*-3 PUFA in FO, induced apoptosis compared to *n*-6 PUFA [138]. *In-vivo*, *n*-3 PUFA have been shown to increase apoptosis in the colon of rats injected with the colon specific

*Portions of this chapter were reprinted with permission from "Dietary fish oil reduces DNA adduct formation while estradiol upregulates apoptosis in response to DNA damage in the rat colon." By Armstrong CM, Allred KF, Allred CD. Dig Dis Sci. 2011 Sep;56(9):2585-94. Copyright 2011 by Springer Science + Business Media, LLC.

carcinogen azoxymethane (AOM) compared to rats fed *n*-6 PUFA [48, 49, 139]. In addition, *n*-3 PUFA are associated with reduced DNA damage in the colon. FO diets have been shown suppress formation of AOM induced DNA adducts in the DNA damaged colonocytes [48, 49]. Thus far, experiments investigating the protective effects of FO and *n*-3 PUFA on DNA damage in the colon have been conducted in male animals and the role of gender has not been fully explored.

A concern that may be raised with male-only models is that there is the potential for sex-specific changes in apoptosis or DNA adduct levels in response to dietary *n*-3 PUFAs. It is possible that estrogen could augment the protective effects of *n*-3 PUFAs. Like *n*-3 PUFAs, estrogen has also been associated with a reduction in colon cancer incidence. Based on data provided by the Women's Health Initiative and other studies, post-menopausal women receiving hormone replacement therapy (HRT) have a decreased likelihood of developing colon cancer as compared to those receiving a placebo [59, 60, 140, 141]. Likewise, estrogen replacement therapy (ERT) has been shown to be effective at reducing colon cancer incidence [142]. Animal studies support these data. Rats treated with estradiol (E_2) had reduced dimethylhydrazine-induced tumor multiplicity [65]. Also, incidence of AOM induced tumors was suppressed in both wild type and estrogen receptor α knockout mice when the animals were fed estrone, a precursor to E_2 [64]. The time during colon cancer development at which estrogenic compounds exert their protective effects, however, is unknown. Previous studies indicate that malignantly transformed colon cancer cell lines are not responsive to treatment with E_2 [63, 66, 68]. This has led to the idea that E_2 is protective during the

initiation and promotion phases of cancer formation. A study fromthis laboratory has shown that in mice treated with AOM, E_2 is protective against the development of high multiplicity aberrant crypt foci (ACF), which are pre-malignant lesions associated with the development of colon tumors. E_2 treatment was also associated with an increase in apoptosis in colonic crypts in the same mice [63]. These data indicate that E_2 is protective during the early stages of colon cancer development and could possibly be effective as early as the point of DNA damage.

While both FO and E_2 are known to be beneficial for preventing colon cancer, the effects of the two together have not been examined. Previously conducted experiments on FO diets have been done in male animals without regard to sex-specific effects that might occur in females. As it stands, it is unknown whether or not FO diets have the same protective capabilities in men and women. It is possible that in the presence of E_2, FO could prove to be more or less beneficial in women than it is in men. In this study, I sought to determine the efficacy of a FO diet in the presence of E_2 by evaluating DNA adduct formation and induction of apoptosis in colonocytes immediately following exposure to carcinogen. Presented data provide a novel understanding of how FO and E_2 protect against colon cancer development which in turn can lead to improved preventative strategies for men and women to reduce the risk of this disease.

Materials and Methods

Animals: Female Sprague-Dawely rats were purchased from Charles River Laboratories at 5-6 wk of age. Rats were singly housed at the Laboratory Animal Resources and Research facility at Texas A&M University. Access to food and water was *ad libitum.* All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Estradiol, diet, and carcinogen treatments: Seven to 10 week old female rats were ovariectomized (OVX) and subcutaneously implanted with either a 2 mg E_2 or cholesterol (vehicle) implant. At the time of OVX, rats were transferred to a phytoestrogen free diet without corn oil (CO) or fish oil (FO). One week following OVX, animals were moved to experimental diets containing either 15% corn oil or 3.5% CO and 11.6% FO with half of each of the E_2 and vehicle groups receiving either diet. Experimental diets were also phytoestrogen free (Pectin Basal Mix, Harlan; Indianapolis, IN). Fresh powdered diet was supplied to the animals each day. Basal diets were identical in vitamin content and each contained 10 g/kg Vitamin E. The FO diet contained an additional 58 ppm Vitamin E. The tissue lipid profiles in the colonic tissue resulting from these diets in rats have been previously characterized by Lee et al. [143]. Three wk following OVX, the animals were sacrificed at four different time points: 0 (no carcinogen), 6, 9, and 12 hr after injection with 15 mg/kg azoxymethane (AOM), a colon specific carcinogen. Each group had an n=5 rats per time point except for 0 hr CO in which n=4. Colons were removed and two 1 cm sections were taken from

the distal end. One section of each was fixed in 4% paraformaldehyde (PFA) for 4 hr, the other in 70% ethanol overnight prior to embedding in paraffin.

Plasma E_2 measurement: Blood was collected via cardiac puncture when the animals were terminated. Plasma was extracted by centrifuging the blood samples at 4°C for 15 min at 2500 RPM. E_2 concentrations were determined in a subset of vehicle and E_2 animals, n=14 from each treatment group, using an Estradiol EIA kit (Cayman; Ann Arbor, MI) according to the manufacturer's instructions. In brief, the samples were diluted 1:10 in enzyme immunoassay buffer and aliquoted into wells. The samples were incubated on the plates with an E_2 linked acetylcholinesterase tracer which serves as a competitor against E_2 present in the plasma sample for binding in the EIA format. The amount of tracer bound is inversely proportional to the E_2 in the sample. The wells were developed with Ellman's reagent for 75 min and absorbance was read on a plate reader at 415 nm. E_2 concentrations were determined by comparison to standards using the formula supplied in the kit.

Immunohistochemistry for O^6-methyldeoxyguanosine adducts: Four micrometer sections were taken from the 70% EtOH fixed tissues. Sections were deparrafinized and rehydrated. Endogenous peroxidase was quenched using 3% H_2O_2 in methanol for 40 min. Antigen retrieval was achieved by microwaving in 10 mM citrate buffer for 10 min. Following washing, slides were incubated with the primary anti-O^6-methyldeoxyguanosine antibody (Squarix Biotechnologies, Germany) at a 1:500 dilution at 4°C overnight. The primary antibody was detected using the labeled streptavidin–biotin method (LSAB2; Dako, Denmark) as per the manufacturer's directions; tissues

were incubated in a humidified chamber for 10 min each of a biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase separated by rinses in PBS. Subsequently, the slides were incubated in 1% diaminobenzidine (DAB) (Sigma-Aldrich; St. Louis, MO) in PBS for 6 min followed by dehydration and coversliping.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed using the ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Millipore; Billerica, MA) following the manufacturer's instructions with slight modifications. Briefly, PFA fixed tissues were rehydrated then treated with 10 μg/mL Proteinase K for 3 min at 37°C. Endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol for 30 min. Tissues were incubated at room temperature (RT) for 20 sec in equilibration buffer followed by 1 hr at 37°C in reaction buffer plus TDT enzyme. Omission of the TDT enzyme was used as a stain control. Next, slides were placed in stopwash solution for 10 min followed by incubation for 30 min at RT in anti-digoxigenin in a humidified chamber. Point five percent DAB for 20 sec was used as the chromagen and nuclei were counter stained in 0.5% methyl green for 5 sec. Lastly, slides were dehydrated and coversliped.

Tissue analysis: Twenty intact crypts were evaluated per animal for each immunohistochemistry stain. Crypts were symmetrically bisected and the right halves of the crypts were further divided into equal thirds along their height to evaluate differences based on crypt cell position. For the O^6-methyldeoxyguanosine (O^6-MedG) DNA adduct analysis, staining intensity of individual nuclei were analyzed using ImageJ, a

program which returned values based on the color intensity of the selected image, and each value was exported into Microsoft Excel. A background value taken from an uninvolved region of the tissue was subtracted from each image to account for non-specific staining within that tissue. The mean staining intensity of the entire crypt for each rat as well as the mean staining intensity for individual tertiles of the crypts was determined and compared across time points.

For apoptosis, the number of positive cells per crypt column was divided by the total number of cells for that column to obtain the proportion of apoptotic cells. The same procedure was used to analyze each tertile. The percentages of apoptotic cells were compared across regions of the crypt and time points after AOM injection.

Statistical analysis: A Student's *t* test was performed for analysis of plasma E_2 data. One-way ANOVA was used for all other outcomes. ANOVA data was run in Minitab 15 and *t* tests were performed in Microsoft Excel. All values are listed as group means with error bars presented as standard error of the mean.

Results

Plasma E_2 concentrations: Plasma E_2 levels were measured in both vehicle control and E_2 treated rats. While some studies have indicated that foods high in Vitamin E, such as FO, may increase E_2 production within an animal, I do not believe this to be the case in this study as diet was seen to have no significant effect on circulating blood E_2 levels. CO fed vehicle treated rats had a mean plasma E_2 concentration of 0.22 nmol/L while FO fed E_2 treated animals had a concentration of

0.21 nmol/L. Rats receiving E_2 had plasma E_2 concentrations of 2.42 nmol/L and 2.47 nmol/L on the CO and FO diets respectively. This level falls within the physiological range for premenopausal women.

Fish oil reduces formation of O^6-methyldeoxyguanosine adducts: Immunohistochemistry was used to evaluate the formation of O^6-MedG adducts in the colon of rats at 0, 6, 9, and 12 hr following injection with AOM (Figure 2.1 A-D). When evaluating the full height of the crypt, FO-fed animals had significantly reduced amounts of DNA adducts compared to CO-fed rats at 9 hr and 12 hr post treatment with AOM (Figure 2.1 E and F). A similar magnitude in the decrease in DNA adducts was observed between diet groups regardless of the presence or absence of E_2. The largest reductions in adduct staining intensity in response to diet were detected at 12 hr for both E_2 and vehicle treated animals. Different populations of cells reside in different locations of the crypt; actively proliferating cells and stem cells are generally located in the bottom third while the top, lumenal, third contains older cells which are about to be sloughed off [144]. To see if there were differences in O^6-MedG staining intensity with regards to cell location in the crypt, each crypt was divided into equal thirds latitudinally. All crypt positions and times after AOM were evaluated; only the statistically significant regions and times are presented. As seen in Table 2.1, significant differences between the two dietary groups were seen in all three thirds of the crypt: bottom, middle, and top, at both 9 hr and 12 hr. In addition, E_2 treated FO-fed rats also had significantly fewer DNA adducts than the E_2 treated CO-fed rats at 6 h post AOM in the bottom third of the crypt. No significant differences were seen between groups

receiving the same diet in response to E_2 treatment in any location of the crypt for any time point (Figure 2.1 G and H).

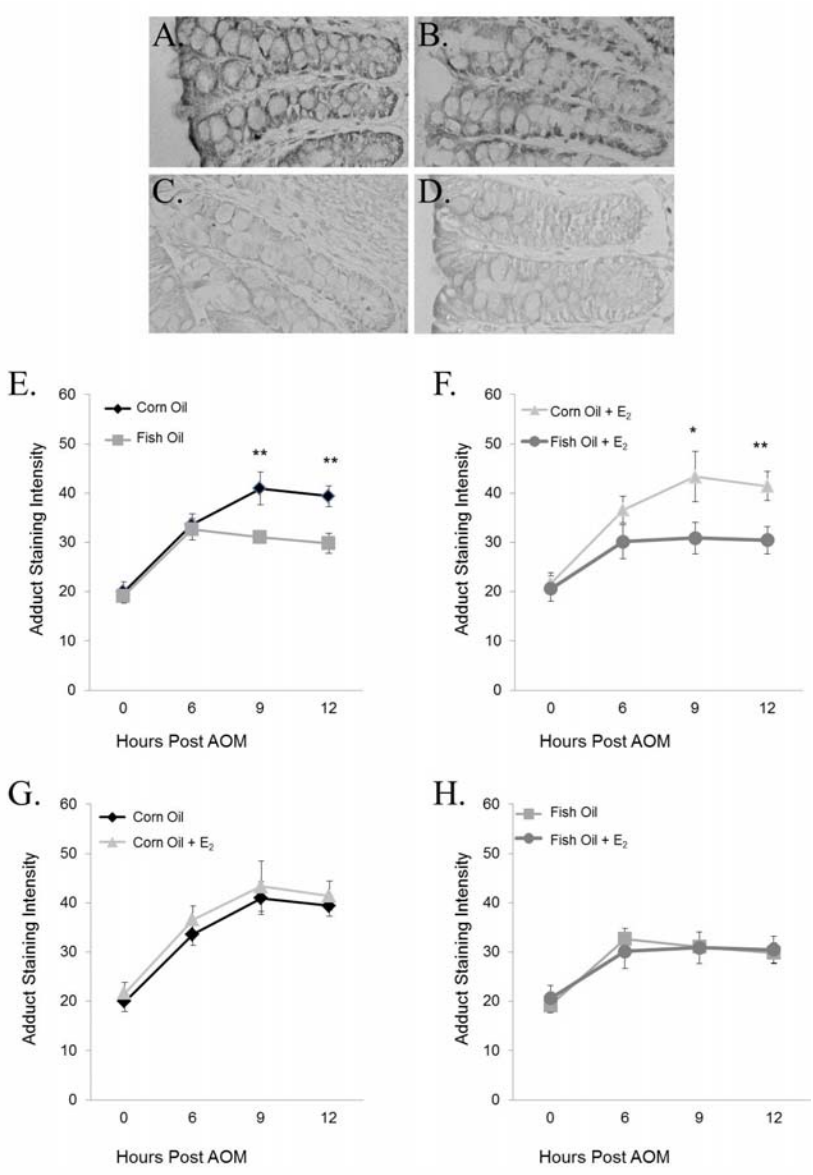

Figure 2.1. Effect of diet and E_2 on staining intensity of O6-MedG DNA adducts. Representative photographs of O6-MedG adduct stained crypts in corn oil (A), corn oil + E_2 (B), fish oil (C), and fish oil + E_2 (D) treated rats 9 h post AOM. (E) Adducts in the colon of corn oil verses fish oil fed rats. (F) Adducts in the colon of corn oil + E_2 verses fish oil + E_2 rats. (G) Adducts in the colon of corn oil verses corn oil + E_2 rats. (H) Adducts in the colon of fish oil verses fish oil + E_2 rats. Data depicted as means ± standard error. * $P<0.05$; ** $P<0.01$ between groups for that time point.

Table 2.1. Significant diet effects on DNA adduct staining intensity by time and location.

Hours post AOM	Crypt Position	Corn oil Adduct Staining Intensity	Fish oil Adduct Staining Intensity	Corn oil + E_2 Adduct Staining Intensity	Fish oil + E_2 Adduct Staining Intensity	P-Value
9	Bottom 1/3	39.93	32.53	-	-	0.0402
9	Middle 1/3	41.33	31.17	-	-	0.0085
9	Top 1/3	41.85	30.00	-	-	0.0062
12	Bottom 1/3	38.60	31.12	-	-	0.0186
12	Middle 1/3	39.92	28.88	-	-	0.0021
12	Top 1/3	39.92	29.40	-	-	0.0061
6	Bottom 1/3	-	-	39.42	29.23	0.0325
9	Bottom 1/3	-	-	44.66	32.62	0.0337
9	Middle 1/3	-	-	43.37	30.73	0.0301
9	Top 1/3	-	-	41.79	29.15	0.0167
12	Bottom 1/3	-	-	42.00	30.25	0.0131
12	Middle 1/3	-	-	42.49	30.88	0.0087
12	Top 1/3	-	-	39.89	30.18	0.0124

Dietary effects on apoptosis: In an effort to determine whether dietary FO in the presence or absence of E_2, could enhance induction of apoptosis in colonocytes, the percentage of apoptotic cells was evaluated using a TUNEL assay in the colon of rats at 0, 6, 9, and 12 hr following injection with AOM (Figure 2.2). For all treatment groups, apoptosis occurred predominately in the bottom third of the crypt with decreasing levels in the middle to the top (Figure 2.3A). A significant overall diet effect was observed at 12 hr post AOM between the vehicle treated groups; FO fed rats had decreased apoptosis as compared to CO fed rats (Figure 2.3B). No differences in apoptosis were observed in response to diet in the E_2 treated rats (Figure 2.3C).

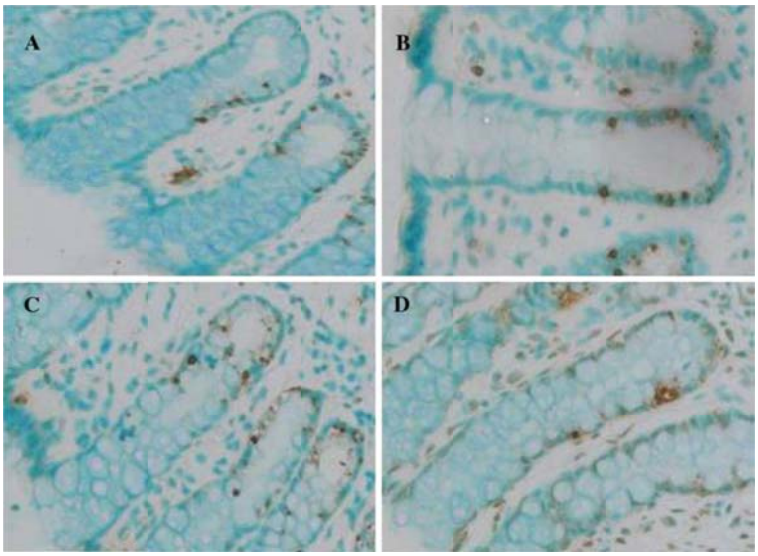

Figure 2.2. Representative photographs of the TUNEL assay. Corn oil (A), corn oil + E_2 (B), fish oil (C) and fish oil + E_2 (D) treated rats 9 h post AOM.

As with the O^6-MedG adduct analysis, the crypts were divided into equal thirds to investigate the differences in apoptosis as it related to cell position within the crypts. Significant diet effects occurred in the bottom third at 9 hr and 12 hr post AOM between the vehicle groups and at 9 hr post AOM in the middle third between the E_2 treated groups. In each case, the FO diet was observed to have reduced apoptosis (Table 2.2).

E_2 effects on apoptosis: Diet independent effects of E_2 on apoptosis in the rat colon were also examined using a TUNEL assay. Overall, E_2 treatment significantly increased apoptosis in both diet groups at 12 hr post AOM (Figure 2.3D and E). All regions and time points following AOM injection were evaluated for significance between groups; significant effects are presented in Table 2.3. When the crypts were divided into thirds, significant increases in apoptosis in response to E_2 were observed in the top third of the colon crypts compared to vehicle animals for both diet groups (Figure

2.3A). When divided out into individual time points, significant E_2 effects were observed in the CO fed rats in the top third of the crypt 12 hr post AOM; E_2 treatment resulted in increased apoptosis over the vehicle animals. The FO fed groups had significant E_2 effects in the bottom and top thirds of the crypt 12 hr post AOM with the E_2 treated groups having increased apoptosis. (Table 2.3).

Table 2.2. Significant effects of diet on apoptosis by cell location and time

Hours post AOM	Crypt Position	Corn oil % Apoptotic	Fish oil % Apoptotic	Corn oil + E_2 % Apoptotic	Fish oil + E_2 % Apoptotic	P-Value
9	Bottom 1/3	15.07	11.18	-	-	0.0484
12	Bottom 1/3	10.41	5.79	-	-	0.0056
9	Middle 1/3	-	-	4.67	1.94	0.0029

Table 2.3. E_2 effects on apoptosis by cell location and time

Hours post AOM	Crypt Position	Corn oil % Apoptotic	Fish oil % Apoptotic	Corn oil + E_2 % Apoptotic	Fish oil + E_2 % Apoptotic	P-Value
12	Top 1/3	0.22	-	1.34	-	0.0432
12	Bottom 1/3	-	5.79	-	9.29	0.0227
12	Top 1/3	-	0.11	-	1.22	0.0202

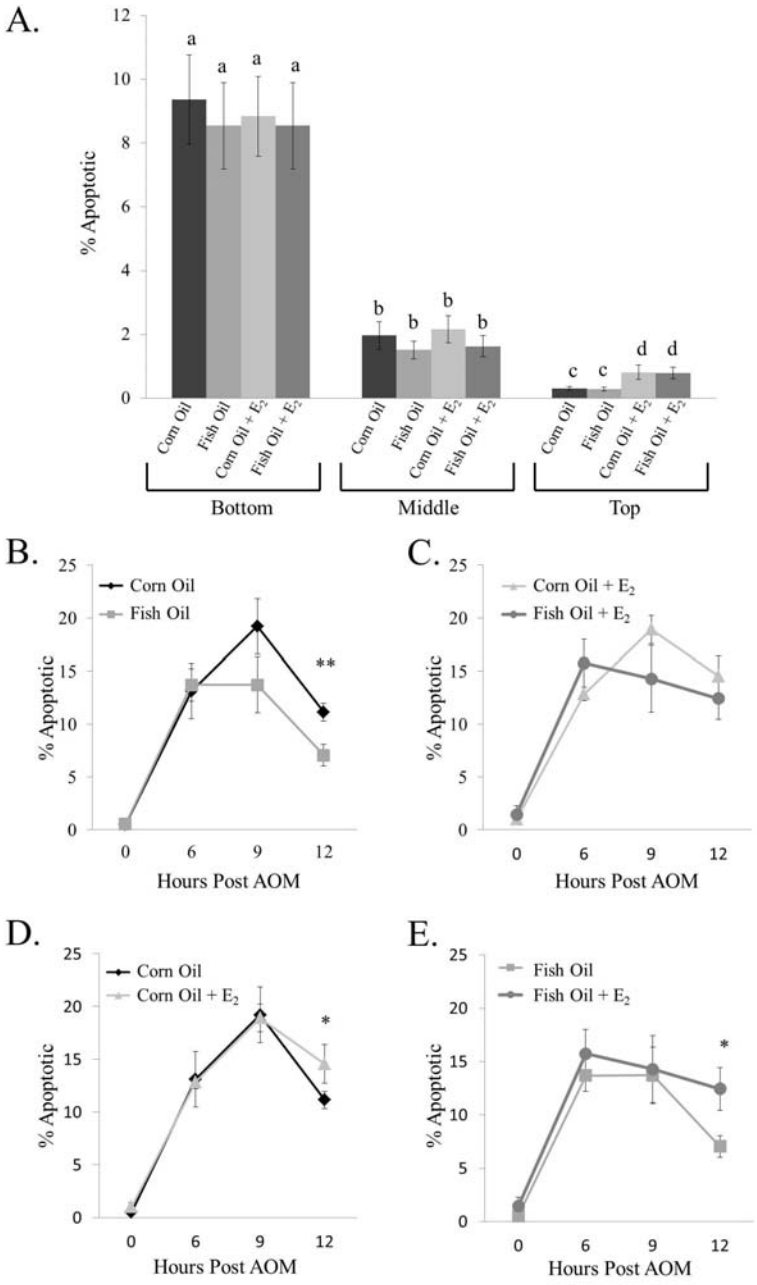

Figure 2.3. Effect of diet and E_2 on apoptosis. (A) Location of apoptosis. Bars without a common letter differ, P<0.01. (B) Apoptosis in the colon of corn oil verses fish oil fed rats. (C) Apoptosis in the colon of corn oil + E_2 verses fish oil + E_2 rats. (D) Apoptosis in the colon of corn oil verses corn oil + E_2 rats. (E) Apoptosis in the colon of fish oil verses fish oil + E_2 rats. Apoptosis in the colon of rats sacrificed at 0, 6, 9, and 12 hr after injection with AOM was analyzed using a TUNEL assay. Data depicted as means ± standard error. * P<0.05; ** P<0.01 between groups for that time point.

Discussion

Evidence exists that both E_2 and FO are protective against the formation of colon cancer [32, 60, 132, 133, 142]. For FO, decreased levels of DNA adducts and an induction of apoptosis have been shown to be mechanisms of action [43, 49, 135, 139]. E_2 treatment is linked to increased apoptosis [145] and reduced numbers of aberrant crypt foci [63]. Thus far, experiments investigating FO diets at the point of DNA damage have been conducted in male animals and the effects of FO and E_2 within the same animal have not been thoroughly examined. Understanding the potential interactions between FO and E_2 could lead to clinically relevant discoveries for dietary recommendations and hormone treatments to better help reduce the risk of colon cancer in the future.

The first part of this study examined the effects of a FO or CO diet in female rats, in the presence or absence of physiological concentrations of E_2, on O^6-MedG DNA adduct formation. O^6-MedG DNA adducts were decreased 9 and 12 hr after AOM injection in rats fed the FO diet compared to the CO diet, both in the vehicle and E_2 treated groups. This observation is in accord with previous studies which have been conducted in male animals [49, 139]. I detected significant reductions in DNA adduct formation with dietary FO 9 and 12 hr following AOM injection with the largest reduction occurring at the 9 hr time point. In regards to adduct staining intensity localization, these data demonstrated that FO fed rats had reduced staining intensity throughout the three tertiles (top, middle and bottom) of the crypt compared to CO fed rats. Collectively, these data suggest that FO is equally protective against O^6-MedG

adduct formation regardless of the presence or absence of E_2. The fact that E_2 does not alter the ability of a diet high in FO to suppress adduct formation is important because it identifies this as a primary mechanism of reducing colon tumor formation in both males and females.

Next I investigated the effects of E_2 on DNA adduct formation independent of diet group to determine if E_2 is protective against DNA damage in the colon. An earlier study in this laboratory demonstrated that while E_2 induces physiological changes in non-malignant colonocytes, these effects are lost following malignant transformation of the same cells [63]. This implicates a role for E_2 in the chemoprevention of colon cancer at the early stages of tumor development. DNA adducts are indicative of DNA damage within a cell which occurs prior to malignant transformation. Phytoestrogens, plant derived compounds known to have estrogenic properties, can affect DNA adduct formation in the colon. One study detected an increase in 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP) induced DNA adducts in the colon of rats fed genistein, a soy derived phytoestrogen, [146] whereas Giri et al. 1995 observed a reduction in DMBA-induced DNA adducts in genistein fed mice [147]. Other studies have examined the role of gender on DNA adduct formation in the colon and have found no change in PhIP induced adducts between male and female rats [148]**.** It is likely that the differing effects of estrogenic compounds on adduct formation in the colon are dependent on the type of adduct being analyzed. When I examined O^6-MedG adduct staining intensity in the colon following AOM exposure with respect to E_2 treatment, no significant differences were seen. This implies that while E_2 is protective against AOM induced

colon carcinogenesis, it is not due to a protection against O^6-MedG DNA adduct formation though these results may be specific to the type of adduct evaluated.

In normal colonic tissue, stem cells at the base of the crypt give rise to epithelial cells [144]. In healthy colon tissue, apoptosis is generally localized to the lumenal region of the crypt and occurs in fully differentiated cells [149]. In response to DNA damage resulting from carcinogen treatment, apoptosis in colonocytes occurs mostly at the base of the crypt near the actively proliferating cells [51]. On average, the largest percentage of apoptotic cells was observed 9 hr post AOM injection in this study. These data coincide with the time point with the highest average staining intensity for O^6-MedG DNA adducts and indicates that the level of apoptosis within the colonic crypts may be directly related to formation of DNA adducts. Prior studies by other groups have seen an overall lack of effect of a FO diet on apoptosis except in the lumenal third of the crypt where the FO diet had significantly increased apoptosis compared to CO fed animals [49]. In contrast to data previously published in male animals, an increase in apoptosis was observed in rats on the CO diet not receiving E_2 12hrs post AOM compared to FO fed rats. This was attributable to increased apoptosis in the bottom third of the colon crypts and was not observed in CO fed animals in the presence of E_2 treatment. The reasons for the contrast between this data and that seen in male animals are not clear and will require further investigation, but it highlights the possibility that *n*-6 PUFAs may have unique physiological effects in postmenopausal women compared to pre-menopausal women with higher circulating levels of E_2. There were no differences seen between diet groups in the lumenal third of the crypts in relation to apoptosis.

Similarly, human trials conducted in both men and women have shown that increased consumption of fatty fish does not induce apoptosis in the colonic crypts [150]. Although apoptosis was not increased in the female animals in this study in response to a FO diet, evidence remains that *n*-3 PUFAs are protective against colon cancer. In women, however, induction of apoptosis by dietary *n*-3 PUFAs may not be a primary method of protection.

When the effects of E_2 on apoptosis were analyzed within the same diet groups, it was seen that E_2 treatment resulted in a significant increase in apoptosis independent of diet at the 12 hr time point following induction of apoptosis with AOM. Another study in this laboratory has demonstrated that apoptosis is upregulated in the colon of mice by E_2 8 wk after AOM exposure at the ACF stage of colon cancer development [63]. This fact, combined with the increase in apoptosis observed in this study, implies a potential up-regulation of apoptosis at the point of DNA damage that persists through later stages of tumor formation. E_2 treatment resulted in significant increases in apoptosis in the lumenal third for both diet groups and the bottom third of the crypts in FO fed animals. The largest increase in apoptosis in response to E_2 occurred in the lumenal third of the crypts. In this region, the levels of apoptosis were still increasing at the 12 hr time point in E_2 treated animals compared to control. This suggests that E_2 can upregulate apoptosis in the lumenal third of crypts in response to DNA damage which is important because these colonocytes are older and as such may be more prone to DNA damage compared to those in the lower regions of crypt. Additionally, E_2 upregulated apoptosis in the bottom third of the crypts in the FO animals; this region is associated with active

proliferation. By inducing apoptosis in this area of the crypt, DNA damaged cells have a reduced chance of proliferating and propagating any DNA damage into new cells. Further study is required to determine whether the levels of apoptosis continue to increase after 12 hr and for how long the levels of apoptosis remain elevated. The observed induction of apoptosis in response to E_2 treatment demonstrates for the first time that E_2 is protective against colon cancer development as early as the point of DNA damage.

In conclusion, these studies indicate that regardless of the estrogen status, a FO diet is effective at reducing O^6-MedG DNA adducts in rats injected with AOM. These data are important because they show that FO diets are likely to be equally protective in men and women against O^6-MedG DNA adduct formation in the colon. In regards to apoptosis, this study is the first to observe that E_2 is capable of upregulating apoptosis in colonic crypts within hours of DNA damage. In conjunction with previous data from this laboratory [63], these data support the idea that E_2 is protective prior to malignant transformation. Specifically, my data demonstrate that E_2 induces apoptosis in colonocytes at the point of DNA damage. Collectively, these data are a critical step in understanding the protective role that *n*-3 PUFAs and E_2 play in lowering colon cancer incidence and will be important in developing chemopreventative strategies for reducing the occurrence of this disease.

CHAPTER III

A NOVEL SHIFT IN ESTROGEN RECEPTOR EXPRESSION OCCURS AS ESTRADIOL SUPPRESSES INFLAMMATION-ASSOCIATED COLON TUMOR FORMATION*

Introduction

Women have a reduced risk for developing colon cancer as compared to men. This protection is lost, however, once a woman reaches menopause, suggesting estrogen as a protective agent against colon tumor development. Data from the Women's Health Initiative (WHI) study as well as other clinical trials have confirmed this idea as it has been documented that post-menopausal women on either hormone replacement therapy (HRT) or estrogen replacement therapy (ERT) have a reduced incidence of developing colon cancer [58-62].

Animal studies have also investigated the role for estrogens in abating colon cancer occurrence: dimethylhydrazine-induced colon tumor number was reduced in rats receiving estradiol (E_2) [65]. Additionally, the multiplicity of tumors induced by azoxymethane (AOM) was suppressed by orally administered estrone, a precursor to E_2, in both estrogen receptor (ER) α knockout (ERαKO) and wild type (WT) mice [151].

*Portions of this chapter were reprinted with permission from "A novel shift in estrogen receptor expression occurs as estradiol suppresses inflammation-associated colon tumor formation" by Armstrong CM, Billimek AR, Allred KF, Sturino JM, Weeks BR, Allred CD. Endocr Relat Cancer. 2013 Jun 27;20(4):515-25. Copyright 2013 by Society for Endocrinology.

Not only has estrogen been linked to reduced incidence of sporadic colon cancer, but data also show that the risk for inflammation-associated colon cancer may be influenced by E_2. Epidemiological studies have demonstrated that women have a lower risk for developing this disease as compared to men which may be due to the presence of estrogens [111]. The mechanism mediating this protection, however, is poorly understood. It is important to investigate this subtype of colon cancer and to develop strategies against the disease because the risk for being diagnosed with colon cancer is increased in persons suffering from inflammatory bowel disease (IBD). Both ulcerative colitis and Crohn's disease are associated with an increased cancer risk [101, 152, 153]. In fact, patients with inflammation-associated colon cancer have a worse prognosis and survival rate compared to those with sporadic colon cancer [154]. The tumors that develop have distinct characteristics compared to those from sporadic colon cancer; they are commonly flat and infiltrating and often occur in multiples. Additionally, there is a higher incidence of high-grade, mucinous carcinomas in inflammation-associated colon cancer. The mechanism linking IBD and colon tumor formation is not yet fully understood but is likely associated with enhanced occurrence of DNA mutations in colonic epithelia due to chronic inflammation.

The focus of the present study was to investigate the timing of when E_2 may be administered and be protective against inflammation-associated colon tumor formation. While the WHI study did find that HRT prevented osteoporosis, reduced fractures, and lowered the incidence of colorectal cancer, the study was ended prematurely due to an observed increase in risk for invasive breast cancer and heart disease. In a follow-up to

the WHI study, it was observed that the protective effect of HRT in protecting against colon cancer was completely lost in as few as 3 years after discontinuing HRT [155]. This indicated that the timing of exposure to E_2 is an important factor when considering E_2 as a chemoprotective agent. By using an experimental protocol in which I delayed E_2 treatment in ovariectomized mice until after colon cancer had been initiated I sought to determine whether E_2 could protect against colon carcinogenesis even after DNA damage had occurred and tumors had been initiated. To do so, I used AOM and dextran sulfate sodium (DSS), an inflammatory reagent which mimics ulcerative colitis in humans. The presented data provide novel insight into the timing of when E_2 exposure is beneficial for colon cancer prevention and implicate a role for E_2 in inflammation-associated colon cancer.

Materials and Methods

Animals: C57Bl6/J mice heterozygous for ERβKO (+/-) were originally obtained from The Jackson Laboratory. The mice were housed at the Laboratory Animal Resources and Research facility at Texas A&M University. Mice were bred to produce WT and ERβKO offspring and genotype was confirmed using genomic tail DNA. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Generation and identification of colon tumors: Female WT and ERβKO mice were ovariectomized and implanted with a 20 mg cholesterol (Sigma-Aldrich; St. Louis, MO) containing pellet subcutaneously on the back of the neck (Figure 3.1A). At the

time of surgery, mice were placed on a semi-purified phytoestrogen-free diet (AIN-76, Lab Supply; Highland Village, TX) and allowed *ad libitum* access to food and water. Two wk following surgery, the mice were injected with a single 12.5 mg/kg injection of AOM (Sigma-Aldrich; St. Louis, MO). Inflammation of the colon was induced 1 week after AOM injection by way of two 6-day regimens of 2.5% dextran sulfate sodium (DSS) solution in the drinking water separated by 2 wk between regimens. A fresh DSS solution was supplied to the mice on days 3 and 5 of each regimen. One week following the final DSS treatment, the cholesterol containing pellets were removed and the mice were randomly assigned to either E_2 or control groups with half of the mice receiving a new 20 mg cholesterol pellet and the other half receiving a pellet composed of 19 mg cholesterol + 1 mg E_2. After 8 more wk, the mice were injected with bromo-deoxyuridine (BrdU) and sacrificed 2 hr later. Blood was collected via cardiac puncture. The colon was resected and individual masses were cassetted and fixed in 4% paraformaldehyde (PFA) for 4 hr. The size of each mass was assessed using calipers. Uninvolved tissue from the most distal region of the colon not containing visible masses was cassetted and fixed as well. Tumors were classified by a board certified pathologist blinded to treatment groups following H&E staining of the sections.

Ovariectomized control mice, both WT and ERβKO, were kept in parallel with the AOM/DSS mice. These mice were handled in the same manner as the tumor bearing mice with the exception of not receiving AOM and DSS. At the time of sacrifice, 1 cm sections were cut from the distal colon, cassetted, and fixed in 4% PFA. Additionally, mucosal scrapings were collected and flash frozen in liquid nitrogen for RNA isolation.

E_2 treatment in non-AOM/DSS mice: A second set of non-AOM/DSS mice were used to observe the effects of E_2 in healthy tissues. Mice were individually housed, randomly assigned to a control or E_2 treatment group based on age and weight, and given a phytoestrogen free diet. All mice were ovariectomized and control animals were given a 20 mg cholesterol pellet while E_2 mice were given a 19 mg cholesterol + 1 mg E_2 pellet implanted subcutaneously on the back of the neck. Sacrifice occurred on day 72. At time of sacrifice, blood was collected through cardiac puncture. The colon was resected and 1cm sections from the distal end were cassetted and fixed in 4% PFA.

Plasma E_2 levels: Whole blood was collected at the time of sacrifice. Plasma was obtained by centrifuging the samples at 2500 RPM for 15 min at 4°C. Plasma E_2 was measured using the Estradiol EIA kit (Cayman; Ann Arbor, MI) according to the manufacturer's instructions. Samples were diluted 1:10 and the reaction was allowed to develop for 75 min. Absorbance was read on a plate reader at 415 nm and final E_2 concentrations were determined by comparison to standards using the formula supplied in the kit.

Immunohistochemistry for ERβ, ERα and BrdU: Four micrometer sections were taken from the PFA fixed tissues. Sections were deparrafinized and rehydrated. Endogenous peroxidase was quenched using 3% H_2O_2 in methanol for 30 min and antigen retrieval was achieved by microwaving in 10 mM citrate buffer for 20 min. Slides were then incubated in the primary antibody, rabbit-anti-ERβ (Pierce; Rockford, IL) diluted 1:50, rabbit-anti-ERα (Santa Cruz Biotechnology; Santa Cruz, CA) diluted 1:50 or anti-BrdU (Roche; Basel, Switzerland) diluted 1:20 at 4°C overnight in a

humidified chamber. During each stain, the primary antibodies were left off of a single slide to serve as a negative control. The following morning, the slides were washed and then incubated in the secondary antibodies, goat-anti-rabbit-HRP (Santa Cruz Biotechnology; Santa Cruz, CA) diluted 1:50 or goat-anti-mouse-HRP (Abcam; Cambridge, MA) diluted 1:250 for the ERβ and BrdU stains respectively. For ERα, the VectaStain ABC Kit (Vector Laboratories; Burlingame, CA) was used for the secondary. A 1% diaminobenzidine (DAB) (Sigma-Aldrich; St. Louis, MO) solution in PBS was used as the chromagen and Meyer's haematoxylin was used as the counter stain. Lastly, slides were dehydrated and coverslipped.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay: The ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Millipore; Billerica, MA) was used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays following the manufacturer's instructions with slight modifications. PFA fixed tissues were rehydrated and treated with 10 μg/mL proteinase K for 3 min at 37°C. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min. Tissues were incubated at room temperature (RT) for 20 sec in equilibration buffer and then incubated for 1 hr at 37°C in reaction buffer plus TDT enzyme. The TDT enzyme was omitted from an individual section as a negative control for the stain. Subsequently, slides were placed in stopwash solution for 10 min followed by 30 min at RT in anti-digoxigenin in a humidified chamber. A 0.5% DAB solution for 20 sec was used as the chromagen and nuclei were counter stained in 0.5% methyl green for 5 sec. Lastly, slides were dehydrated and coverslipped.

Immunohistochemistry analysis: The proliferation and apoptosis stains were analyzed in the same manner. In the uninvolved tissue (the most distal region of the colon not containing a mass), crypts were symmetrically bisected and the right halves of 20 crypts were analyzed per animal. The total number of positively stained cells was divided by the total number of cells in the crypt column to generate the percentage of apoptotic or proliferative cells for each column. For the tumor sections, 250 cells from four distinct regions of each tumor were analyzed. Analysis of the ERβ and ERα stains was conducted using the image analysis software, Cell Profiler, in conjunction with HKCellCounter, a pipeline designed to specifically identify nuclei within a designated region and return values based on the staining intensity of each identified nuclei. The pipeline identifies nuclei based on parameters, such as size and shape, set by the user within a specified area of a photograph. Twenty intact crypts were analyzed for each uninvolved and non-AOM/DSS sample. Four images of distinct locations from each tumor were analyzed to determine expression levels in the adenocarcinomas. The same parameters were used for each photomicrograph to ensure consistency between analyses.

rtPCR for ERα: RNA was isolated using trizol from tumors in the tumor bearing mice and the mucosal scrapings in the non AOM/DSS treated mice. cDNA was produced from the RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche; Basel, Switzerland) as per the manufacturer's instructions: 1 μL of the diluted sample was mixed with a random hexamer primer, an anchored-oligo$(dT)_{18}$ primer and water. Samples were then placed in a thermal cycler for 10 min at 65°C. Next, reaction buffer, RNAse inhibitor, deoxynucleotide mix, and reverse transcriptase were added to the

sample and then returned to the thermal cycler for 10 min at 25°C followed by 60 min at 50°C and 85°C for 5 min. Samples were then stored at -20°C.

The SYBR Green method was used to assess mRNA content of the samples. Primers against ERα and 18s sequences were produced by Sigma-Aldrich with the following sequences: ERα-Forward; 5'-GACCAGATGGTCAGTGCCTT, ERα-Reverse; 5'-ACTCGAGAAGGTGGACCTGA, 18s-Forward; 5'-TCAAGAACGAAAGTCGGAGGT, 18s-Reverse; 5'-GGACATCTAAGGGCATCACAG. Two microliters of cDNA were mixed with 2.5 μL of forward and reverse primer, 9.5 μL SYBR green and 11 μL water. Prepared samples were loaded into 96 well reaction plates and run for 45 cycles. Samples were run in triplicate. ERβ RNA expression was not analyzed due to the previously collected protein data for ERβ.

Statistical analysis: The tumor number outcome was analyzed using a bootstrap with 1000 replications, a statistical test that compares the medians of groups. *In vivo* apoptosis data was analyzed using a Poisson regression model allowing for over-dispersion and statistical inference was carried out by testing model coefficients corresponding to group comparisons with control using a Wald test. One-way ANOVA was used for the remaining outcomes. ANOVA was run using Minitab 15 and the bootstrap, Poisson regression and Wald test were performed using the statistical software 'R'.

Results

Plasma E_2 concentrations: Plasma E_2 concentrations were measured in all mice. WT and ERβKO mice receiving E_2 pellets both averaged 0.8 nmol/L which is marginally lower than peak E_2 concentrations in a premenopausal non-pregnant woman and well below the levels experienced in pregnancy [156, 157]. Non-ovariectomized mice reach peak E_2 levels during estrus at around 0.24 nmol/L [158]. Animals not receiving E_2 had mean plasma E_2 concentrations of 0.03 nmol/L and 0.04 nmol/L for WT and ERβKO mice respectively. These concentrations are less than half the concentration of plasma E_2 associated with postmenopausal women [159].

Tumor multiplicity and area is reduced with E_2 treatment: To determine whether administration of E_2 following initiation of DNA damage could decrease the formation of colon tumors, colon tumor multiplicity and area were measured in both WT and ERβKO mice following the study design described (Figure 3.1A). Tumors were characterized by a board certified pathologist blinded to treatment groups. The median number of tumors per animal was decreased by nearly 50% in the presence of E_2 in the WT mice ($P = 0.05$) (Figure 3.1B). In comparison, no significant change in median tumor number was observed in the ERβKO mice. In the absence of E_2, ERβKO mice had significantly reduced tumor number compared to WT controls ($P = 0.05$). In regards to the mean size of each tumor, E_2 treatment resulted in a 30% reduction in tumor area in the WT mice ($P = 0.031$). Again, the response to E_2 was not observed in the ERβKO mice. Both control and E_2 treated ERβKO mice had a significant reduction in tumor size compared to WT control mice ($P= 0.001$ and $P = 0.003$) (Figure 3.1C).

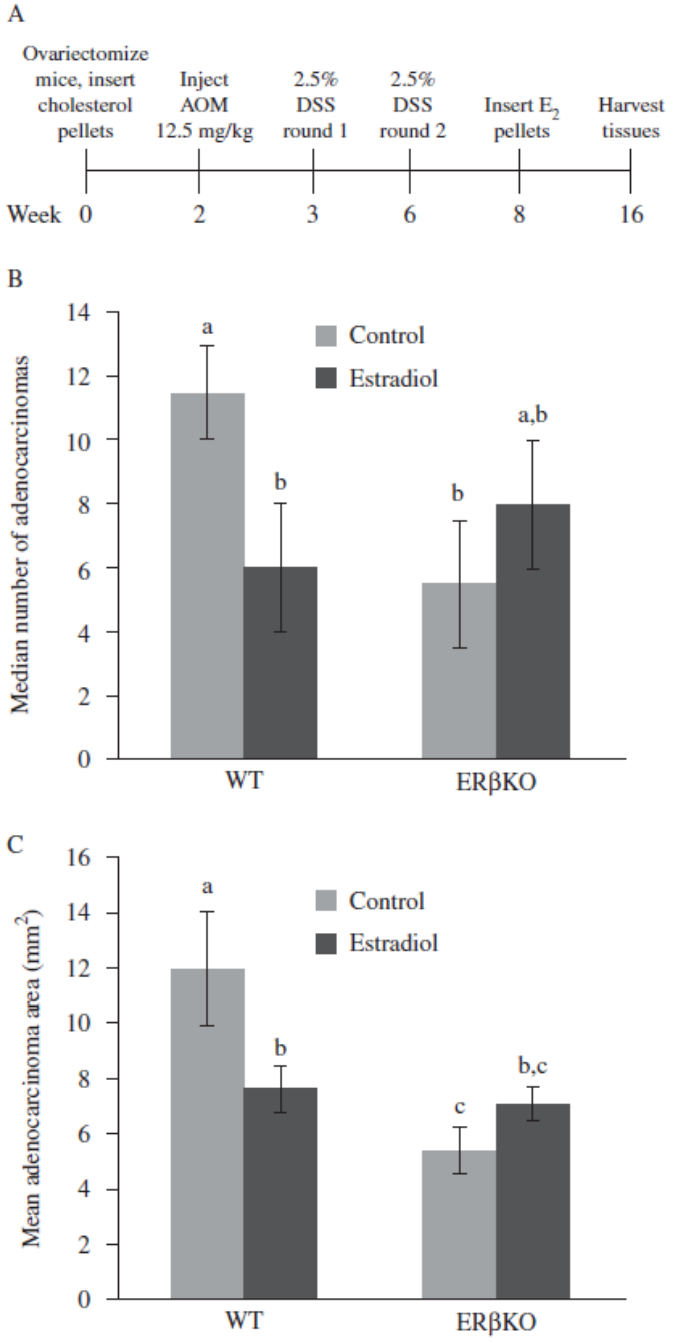

Figure 3.1. Effect of E_2 on tumor number and area. (A) Study design. (B) Median number of tumors per mouse +/- median absolute deviation. (C) Mean area of tumors ± SEM., n=6 mice in the WT and ERβKO control groups, n=7 mice in the WT E_2 group and n=5 mice in the ERβKO E_2 group. Bars without a common letter differ, $P<0.05$.

Cellular proliferation increases in WT E_2 treated mice at the tumor stage: Proliferation of epithelial cells within the tumors and uninvolved colonic crypts was analyzed using immunohistochemistry for BrdU in order to investigate potential mechanisms for the reduction in tumor multiplicity and size observed in the E_2 treated WT mice. Surprisingly, E_2 treatment resulted in significantly increased proliferation in both the uninvolved tissue and the adenocarcinomas in WT mice compared to non-E_2 treated controls ($P = 0.043$ and $P = 0.017$ respectively) (Figure 3.2A-B). In the uninvolved WT tissue, E_2 roughly doubled the amount of proliferation compared to non-E_2 treated mice. In the adenocarcinomas there was a 30% increase in proliferation.

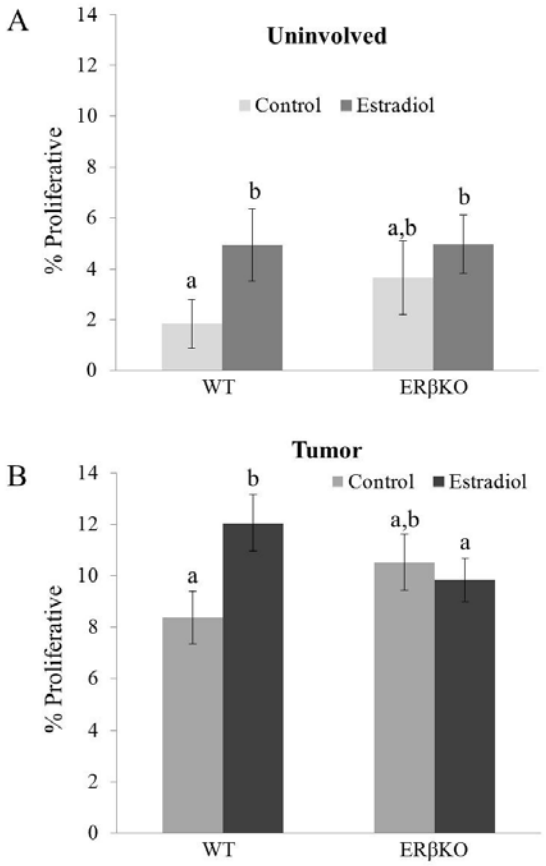

Figure 3.2. Colonocyte proliferation was evaluated in the tumors and uninvolved colon tissue of WT and ERβKO mice using immunohistochemistry. (A) Proliferation in uninvolved colon tissue and (B) proliferation in the tumors. Values are mean percent of positively stained cells ± SEM, n=6 animals in the WT and ERβKO uninvolved control groups, n=7 animals in the uninvolved WT E_2 group and n=5 in the uninvolved ERβKO E_2 group. In B, n=6 mice in the WT control group, n=7 WT E_2 mice, n=6 ERβKO control mice and n=5 ERβKO E_2 mice. Bars without a common letter differ, $P \leq 0.05$.

Apoptosis is down regulated by E_2 at the tumor stage in WT mice: Having observed increases in proliferation in response to E_2, levels of apoptosis were also evaluated in the adenocarcinomas and uninvolved tissue using a TUNEL assay. No significant differences were observed in the percentage of apoptotic cells in uninvolved tissues in response to E_2 treatment for either WT or ERβKO mice (Figure 3.3A). In the

adenocarcinomas, E_2 treatment resulted in a 25% decrease in apoptosis in WT mice (P = 0.039) and no significant difference in ERβKO mice (Figure 3.3B). Contrarily, in the non-AOM/DSS treated mice, the presence of E_2 was correlated with increased apoptosis in the colonic crypts and no changes in proliferation (Figure 3.4A-B).

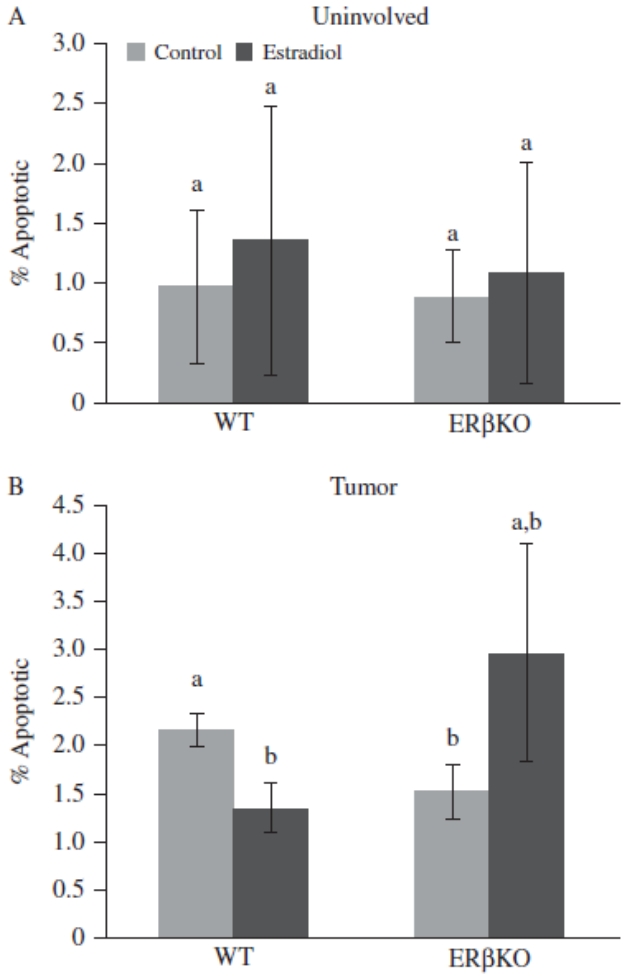

Figure 3.3. Effect of E_2 on Apoptosis. Cellular apoptosis was evaluated in the tumors and uninvolved colon tissue of WT and ERβKO mice using a TUNEL assay. (A) apoptosis in uninvolved colon tissue and (B) apoptosis in the tumors. Values are mean percent of positively stained cells ± SEM, n=6 animals in the WT and ERβKO uninvolved control groups, n=7 animals in the uninvolved WT E_2 group and n=5 in the uninvolved ERβKO E_2 group. In B, n=6 mice in the WT control group, n=7 WT E_2 mice, n=6 ERβKO control mice and n=5 ERβKO E_2 mice. Bars without a common letter differ, $P \leq 0.05$.

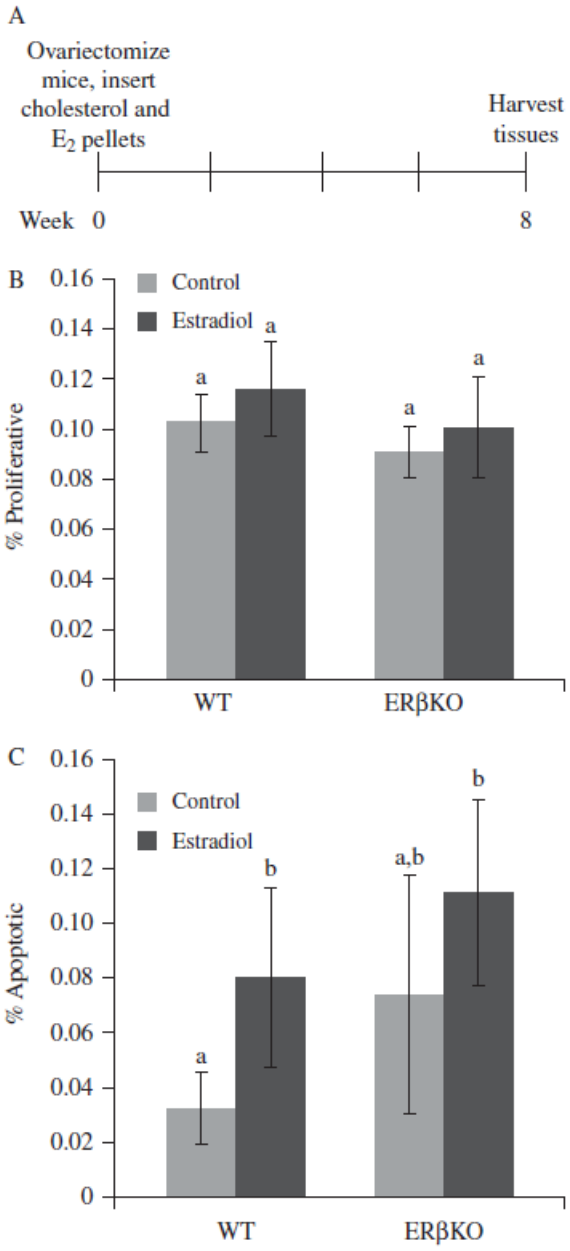

Figure 3.4. Colonocyte proliferation and apoptosis were evaluated in non-diseased WT and ERβKO mice. (A) Study design. (B) Percent of proliferative cells in the colonic crypts ± SEM. (C) Percent of apoptotic cells in the colonic crypts +/- SEM. n=10 mice for each of the WT control and ERβKO E_2 groups and n=8 mice for the WT E_2 and ERβKO control groups. Bars without a common letter differ, $P \leq 0.05$. Data collected by Autumn Billimek [73].

ERβ protein expression declines as tumors develop: In order to investigate physiological changes associated with the observed reduction in tumor area and number in response to E_2, ER expression levels were measured. ERβ was evaluated using immunohistochemistry to observe the effects of inflammation-associated carcinogenesis on protein levels of ERβ in WT mice (Figure 3.5). Tumor sections were compared to uninvolved sections from tumor bearing mice as well as to normal tissue from normal, non-AOM/DSS treated mice from the same animal experiment. Normal colon tissues had the highest staining intensity for ERβ. Uninvolved sections of colon had a significant reduction in staining intensity compared to normal tissues ($P = 0.0007$ and $P = 0.002$ for control and E_2 treated respectively). Analysis of the tumor sections demonstrated even further reduction in staining intensity when compared to non-AOM/DSS tissues and levels were significantly lower in the tumor when compared to uninvolved tissue samples from the same animal ($P < 0.0001$). The reduction in ERβ expression concentrations was not affected by the presence of E_2.

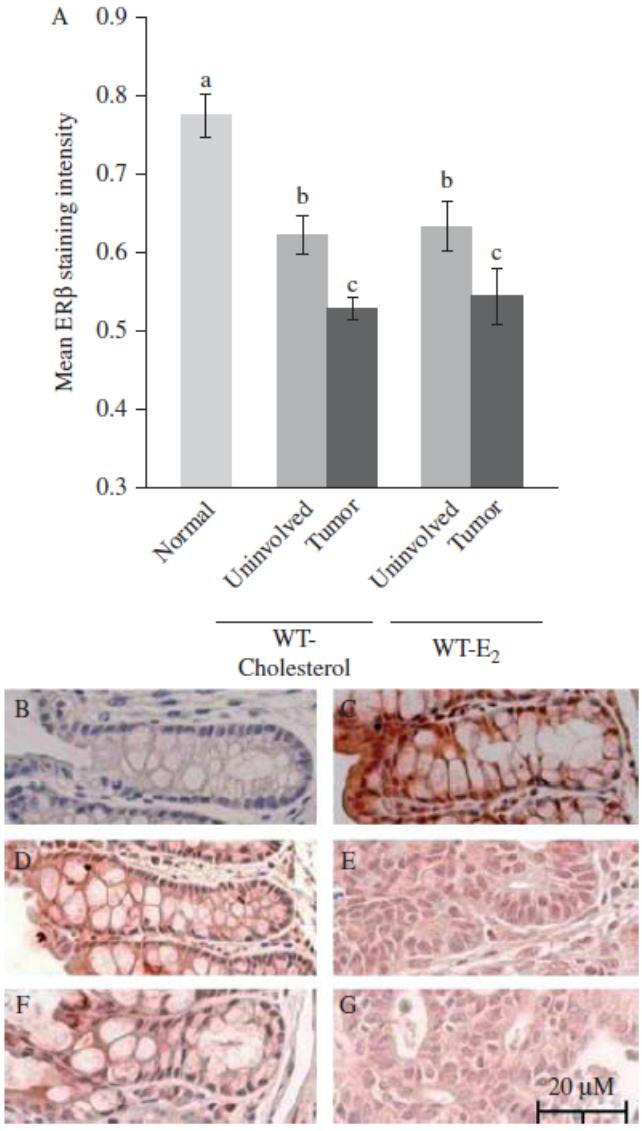

Figure 3.5. ERβ expression was evaluated in the tumors, uninvolved, and normal colon tissue of WT mice using immunohistochemistry. (A) Quantification of staining intensity. Values are mean staining intensity of the tissues ± SEM, n=7 mice in the normal, non-AOM/DSS group, n=6 mice for WT cholesterol groups and n=7 mice in the WT E_2 groups. Bars without a common letter differ, $P \leq 0.0001$. *B-G,* Representative photographs of staining for ERβ in an ERβKO colon (B), normal, non-AOM/DSS treated WT (C), uninvolved WT-control (D), WT-control tumor (E), WT-E_2 uninvolved (F), and WT-E_2 tumor tissues (G).

ERα mRNA and protein expression increases as tumors develop: In addition to measuring ERβ, ERα was also assessed using rtPCR in the tumor and non-AOM/DSS colon tissues. mRNA collected from mucosal scrapings from non-AOM/DSS treated mice in the same animal experiment was compared to mRNA from tumor samples to assess if ERα expression was altered as the tissue progressed to a malignant state (Figure 3.6A). There was a 300-fold increase in ERα expression in the tumors compared to normal mucosa in WT mice not receiving E_2 ($P = 0.021$). Adenocarcinomas from ERβKO mice not receiving E_2 had a 200-fold increase in ERα mRNA expression compared to mucosal scrapings from non-DSS/AOM treated ERβKO mice ($P = 0.042$). In WT E_2 treated mice, the tumors had a 14-fold increase in ERα mRNA expression compared to WT E_2 normal mucosa ($P = 0.015$). In the presence of E_2, ERα mRNA increased 13-fold in ERβKO adenocarcinomas over ERβKO normal mucosa ($P = 0.022$).

Protein expression levels of ERα were evaluated using immunohistochemistry to observe whether or not the mRNA expression levels were representative of protein ERα expression (Figure 3.6B-I). Immunohistochemistry for ERα from tumor sections showed strong positive staining for ERα in WT mice with reduced staining levels in ERβKO mice both with and without E_2 treatment while no staining was evident in the normal tissues for either genotype.

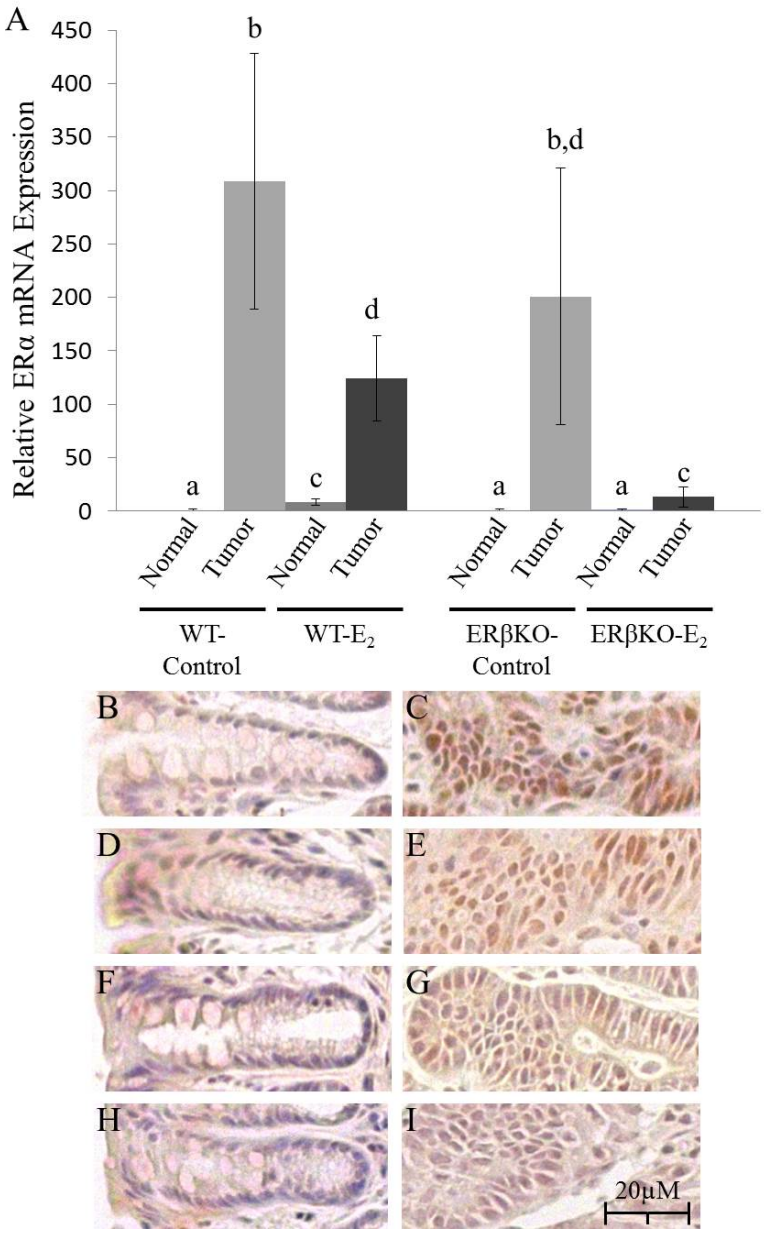

Figure 3.6. **(**A) ERα mRNA expression was evaluated in the tumors and normal colon tissue of mice using rtPCR. Values are mean fold change from normal, non-AOM/DSS WT-control mice +/- SEM, n=7 in the normal groups, n=4 mice for WT-control, n=4 mice in the WT-E_2 tumor group, n=4 mice in the ERβKO-control group n=2 mice in the ERβKO-E_2 tumor group. Bars without a common letter differ, $P \leq 0.0001$. *B-I*, Representative photographs of immunohistochemistry for ERα protein in WT-control (B-C), WT-E_2 (D-E), ERβKO-control (F-G) and ERβKO-E_2 (H-I) mice. For each pair, the normal tissue is on the left and the tumor section is on the right.

Discussion

Studies in both humans and animal models have demonstrated the efficacy for E_2 in protecting against sporadic colon cancer and yet there has been little investigation into the influence of E_2 on inflammation-associated colon cancer. Existing data examining human populations suggest that the presence of E_2 may result in a reduction in the occurrence of this disease [111]. Until this point, no animal studies had investigated the mechanisms involved in the reduction in inflammation-associated colon cancer incidence observed in the presence of E_2. Additionally, the exact timing of when E_2 can be administered and prove protective against colon cancer remains unknown. Previous studies from our laboratory have indicated that E_2 guards against early markers of colon tumor formation. As early as hours following carcinogen exposure at the point of initial DNA damage, E_2 significantly induced apoptosis in the colonic epithelium of rats when compared to control animals [160]. In addition, this laboratory has demonstrated that E_2 suppresses the formation of pre-malignant lesions in the colon that was again associated with an upregulation of apoptosis in colonic epithelia [63].

To date, *in vivo* studies of the protective effects of E_2 during the process of colon carcinogenesis have only investigated continuous E_2 exposure for the duration of the studies. In contrast, the present study aimed to elucidate the chemoprotective role of E_2 when the intervention is given following initiating events in colonic carcinogenesis. The data herein indicates, for the first time, that E_2 is protective prior to the formation of colon tumors even when administered after DNA and inflammatory damage had

occurred in the colon. This protection is characterized by the reduction in tumor multiplicity and area.

After quantifying fewer colon tumors in response to E_2 in this model, I sought to determine the physiological responses to E_2 at the cellular level and how these reactions change over the progression of the disease. In this study, despite the overall decline of tumor number and area, increased proliferation and decreased apoptosis in the colonocytes of WT E_2 treated tumor bearing animals as compared to cholesterol treated controls was observed. In ERβKO mice, there was no change in proliferation between cholesterol and E_2 treated animals for either the uninvolved or colon tumor samples. When apoptosis was evaluated in the tumors of ERβKO mice, E_2 treatment resulted in no significant change compared to the cholesterol treated ERβKO mice. Similarly, data from human trials observing the effects of combined estrogen and progestin therapy in post-menopausal women show that the combined HRT reduced the incidence of colon tumors in these women, however, the tumors that were detected were at a more advanced stage [161]. The fact that this similar phenomenon was observed in human tissues supports the results detected in the mice in the presented study. While the mechanism behind the higher grade tumors was not investigated in the human study, it is possible that it could be similar to what was seen in the mice.

Having observed these counterintuitive responses to E_2 in the tumors and uninvolved tissues in the mice, a second animal study was conducted to investigate how E_2 affects normal colonic tissue. In animals that received neither AOM nor DSS, E_2 treatment resulted in no change in proliferation for either WT or ERβKO mice and the

non-diseased WT mice had increased apoptosis in the colon. These data coincide with the results observed previously in our laboratory and indicate that the response to E_2 may be dependent on the stage of the disease and, possibly, the experimental model used in this study.

An intriguing explanation for the surprising response to E_2 observed for proliferation and apoptosis in the tumor bearing animals could be the changes in ER expression detected in this experiment. Previous studies by this laboratory and others have indicated that ERβ, the primary ER within the colon, is the form of the receptor through which E_2 exerts its protective effects [63, 151]. Other groups have shown that ERβKO mice are more susceptible to inflammation-associated colon cancer than WT mice [113]. Clinical and experimental models of colon carcinogenesis have suggested that protein expression of ERβ decreases as the tissue progresses from normal colonic epithelia to a malignant state [87, 88, 162, 163]. In fact, humans with colon tumors not expressing ERβ are likely to be in more advanced stages of cancer and have a higher risk for death compared to those with tumors in which ERβ is present [89]. In the present study, the ERβ expression levels suggest that a similar trend occurs during the formation of inflammation-associated colon tumors. Normal colonic tissue had the highest staining intensity for ERβ while the tumors had the least, with no changes in ERβ expression due to the presence or absence of E_2. Analysis of the uninvolved tissues revealed ERβ intensity levels between those of the normal and tumor samples. The intermediate staining intensity of the uninvolved tissues is not surprising when one takes into account that while these samples were not cancerous, they were collected from the same colons

as the tumors and as such had also sustained high levels of damage and cannot be considered normal. The varying level of ERβ expression between the tissues indicates that the loss of expression occurs gradually as inflammation-associated colon tumors develop.

In systems where ERα is the predominantly expressed ER, such as the mammary gland, the presence of E_2 is often associated with an increase in cancer growth [74, 75, 164-166]. In normal colon tissue, ERα protein expression is very low, as can be seen by the lack of a positive stain in the correlative tissues in this study. In contrast, adenocarcinomas exhibited positive protein staining for ERα and had significantly increased ERα mRNA expression: as high as 300-fold over control tissues, supporting the idea that ERα activity could be increased in the colon tumors. Contrarily, studies conducted utilizing the APCmin/+ mouse model of intestinal carcinogenesis suggest that loss of ERα was detrimental to the colon [167, 168]. Disparities observed are likely the result of underlying differences between the APCmin/+ mouse model and the inflammation protocol used here.

The possibility of ERα activity exceeding ERβ activity could explain the change in physiological response to E_2 seen in the tumor bearing mice compared to the non-diseased mice and to previous studies in this laboratory observing earlier endpoints in the process of colon carcinogenesis. It is possible that the protection against colon tumor formation in response to E_2 observed in this study occurred before ERβ expression decreased and ERα expression increased, thus delaying the growth of colon tumors.

Once ERα started to be expressed the tumor physiology was altered as indicated by the increased proliferation and decreased apoptosis.

In addition, previous studies from this laboratory suggest that p53 may be a primary modulator of the chemoprotective effect of E_2 on colon carcinogenesis; non-malignant Young Adult Mouse Colonocytes (YAMCs) treated *in vitro* with E_2 had increased apoptosis and an overall reduction in cell number. In YAMCs lacking functional p53, the effect of E_2 was lost [69]. Mutations in *p53* occur in roughly 50% of colon tumors [169] and the loss of p53 functionality could contribute to the change in physiological response to E_2 in the tumors in this study. Due to the timing of tissue collection in the presented experiment, analysis of p53 would be inappropriate due to the loss of proper p53 function observed in most colon tumors. Had tissues been harvested at an earlier time point following AOM/DSS exposure, it is likely a different response to E_2 and relevant p53 signaling would have been observed. However, the presented data are critical in highlighting the complex role of estrogens in the colon.

The current study takes a critical step toward understanding the role of E_2 in colon carcinogenesis. These data demonstrate that E_2 is protective against inflammation-associated colon tumor formation, which is important because it is estimated that up to 396/100,000 people worldwide suffer from IBD, increasing their risk for developing colon cancer [170]. Additionally, E_2 protected against the development of tumors even when introduced to the system following the initiation of inflammation and DNA damage to the colonic tissue. This observation implies that intervention with estrogen therapy may be beneficial in patients considered at high risk

for colon cancer even if they have been menopausal for some time. Of utmost interest in this study was the observed upregulation of ERα expression with a concurrent reduction in ERβ expression. To my knowledge, this study is the first to definitively demonstrate a concurrent and inverse shift in the expressions of ERα and β in any system. Several studies have observed changes in ER expression as the result of treatments or changes in disease state. However, in these studies either only one of the ERs was affected or both receptors had either increased or decreased expression [167, 171]. These data provide new insight into the role of estrogens in reducing colon tumor formation in patients who suffer from colonic inflammation and suggest that intervention can still be successful even if begun later in the tumor development process.

CHAPTER IV

ESTRADIOL PROTECTS AGAINST ACUTE TNBS INDUCED INFLAMMATION IN THE COLON OF MICE

Introduction

Inflammatory bowel disease (IBD) affects over one million people in the United States. Typically, IBD occurs in persons in their twenties and thirties at which point symptoms lesson for a number of years followed by flare-ups of the disease later in life. The two primary forms of IBD, Crohn's disease and ulcerative colitis, both confer a greater lifetime risk for developing colon cancer [101, 102]. This increased risk is most likely due to an increased rate of growth and potential mutation of colonocytes due to the exposure to pro-inflammatory cytokines. Interestingly, women have been observed to be 60% less likely than men to develop inflammation-associated colon cancer, suggesting that female hormones may play a role in the prevention of this disease [111].

One way that estradiol (E_2), the primary form of estrogen in the human body, may be protecting against inflammation-associated colon carcinogenesis is through the modulation of intestinal inflammation. Epidemiological studies have observed that pre-menopausal female patients with Crohn's disease reported a worsening of their IBD related symptoms during menses; the time during the estrous cycle that E_2 concentrations are at their lowest [112]. Not only does this suggest that E_2 could be protecting against intestinal inflammation, but the fact that the same worsening of

symptoms was not observed in women with ulcerative colitis suggests that the role of E_2 in protecting against inflammation could be dependent on the subtype of IBD.

Experimental data to support the protective effects of E_2 on the development of inflammation-associated colon tumors, however, is conflicting. Data from our laboratory indicates that treatment with E_2 in mice following induction of inflammation-associated colon cancer using co-treatment with the colon specific carcinogen azoxymethane (AOM) and dextrtan sulfate sodium (DSS) was effective at reducing both the number and size of colon tumors [72]. Other groups, however, have observed that in mice pre-treated with E_2 and then subjected to AOM/DSS, polyp number and size was increased [114].

The disparity in the observed results is not limited to the tumor stage. Studies looking at the effects of E_2 solely on inflammation have also not reached a consensus. Here though, the incongruent findings are likely the result of the models of inflammation used. Studies using DSS as the inflammatory agent found that E_2 worsened disease severity whereas dinitrobenzene sulfonic acid colitis was improved in the presence of E_2 [114, 115]. Another model of inflammation, 2,4,6-Trinitrobenzenesulfonic acid (TNBS) has also been suppressed by estradiol benzoate and the phytoestrogen genistein [116, 117]. DSS and TNBS induce inflammation using distinct mechanisms resulting in colitis that resembles ulcerative colitis and Crohn's disease respectively and the differential responses to estrogenic compounds to each further suggests that Crohn's disease and ulcerative colitis respond differently to E_2.

The focus of the present study is to investigate the physiological actions of E_2 during acute TNBS colitis in wild type and estrogen receptor (ER) β knockout (ERβKO) mice. While previous studies suggest that estrogen is protective against TNBS colitis, the mechanism behind this protection is still poorly understood. Studies in our laboratory in both sporadic and inflammation-associated colon cancer suggest that ERβ, the primary ER in the colon, mediates the protective effect of E_2 in the colon [63, 72]. To date, however, the role of ERβ in acute TNBS colitis is unknown. Understanding the role of E_2 during acute inflammation is important for developing protective strategies against both Crohn's Disease and inflammation-associated colon cancer.

Methods and Materials

Animals: C57Bl6/J mice heterozygous for ERβKO (+/-) were originally obtained from The Jackson Laboratory. The mice were housed at the Laboratory Animal Resources and Research facility at Texas A&M University. Mice were bred to produce WT and ERβKO offspring and genotype was confirmed using genomic tail DNA. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Induction of colitis: Female mice were ovariectomized (OVX), implanted with either a 20 mg cholesterol containing pellet or a 0.5 mg E_2 + 19.5 mg cholesterol (Sigma-Aldrich; St. Louis, MO) pellet and placed on a phytoestrogen free diet. Two wk later they were pre-treated with 1% TNBS dermal absorption through the skin on their backs. One week following pre-treatment, a 2.5% TNBS solution was administered

intrarectally 2-4 cm into the colon using a flexible plastic gavage tube (Instech Solomon) after a 12 hr fast. Mice were sacrificed 5 d post intrarectal TNBS. Colons were resected and latitudinaly bisected. One half of the colon was rolled into a Swiss roll and fixed in PFA. The other half was snap frozen in liquid nitrogen for cytokine analysis. Inflammation and injury in the colons were assessed by a board certified pathologist on H&E stained colon Swiss rolls on a scale of 0-3. In brief, a score of 0 would indicate no inflammation or injury noted while a score of 3 would indicate severe inflammation or injury.

Cytokine multiplex analysis: Snap frozen colon tissues were homogenized in 1 mL Tissue Protein Extraction Reagent (Thermo Scientific.) Total protein content was assessed using the *DC* Protein Assay (Bio-Rad) as per the manufacturer's instructions. The Magnetic Mouse Cytokine/Chemokine Milliplex Map Kit was used to measure tissue levels of IL-6, IL-10, IL-12(p40), IL-17, GM-CSF, IFNγ, MCP-1, MIP-1α, and TNFα following the manufacturer's instructions. All samples were diluted to 10 M. In brief, 25 µL diluted sample was added per well of a 96-well plate in addition to 25 µL assay buffer and 25 µL of the magnetic beads provided in the kit. The plate was sealed and agitated on a plate shaker overnight at 4°C. The following day, the 96-well plate was placed on a hand-held magnet, the contents of the wells removed, and then the plate was washed twice. Following washing, 25 µL of detection antibodies were added to each well and the plate was incubated at room temperature for 1 hr with agitation. Next, 25 µL of Streptavidin-Phycoerythrin were added to each well the plate was incubated at room temperature for half an hour with agitation. The plate was then washed twice, the

magnetic beads resuspended in 150 µL Luminex Sheath Fluid, and the plate was run on a Luminex 200.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay: Paraffin embedded, paraformaldehyde (PFA) fixed colon Swiss-rolls were rehydrated and the ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Millipore; Billerica, MA) was used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays following the manufacturer's instructions with slight modifications. PFA fixed tissues were rehydrated and treated with 10 µg/mL proteinase K for 3 min at 37°C. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min. Tissues were incubated at room temperature (RT) for 20 sec in equilibration buffer and then incubated for 1 hr at 37°C in reaction buffer plus TDT enzyme. The TDT enzyme was omitted from an individual section as a negative control for the stain. Subsequently, slides were placed in stopwash solution for 10 min followed by 30 min at RT in anti-digoxigenin in a humidified chamber. A 0.5% DAB solution for 20 sec was used as the chromagen and nuclei were counter stained in 0.5% methyl green for 5 sec. Lastly, slides were dehydrated and coverslipped.

Immunohistochemistry for BrdU: Four micrometer sections were taken from the PFA fixed tissues. Sections were deparrafinized and rehydrated. Endogenous peroxidase was quenched using 3% H_2O_2 in methanol for 30 min and antigen retrieval was achieved by microwaving in 10 mM citrate buffer for 20 min. Slides were then incubated in the primary antibody, anti-BrdU (Roche; Basel, Switzerland) diluted 1:20 at 4°C overnight in a humidified chamber. During each stain, the primary antibody was

left off of a single slide to serve as a negative control. The following morning, the slides were washed and then incubated in the secondary antibody, goat-anti-mouse-HRP (Abcam; Cambridge, MA) diluted 1:250. Meyer's haematoxylin was used as the counter stain. Lastly, slides were dehydrated and coverslipped.

Immunohistochemistry analysis: The TUNEL and BrdU assays were analyzed in the same manner. For each, 20 well oriented crypts per animal from both the distal and proximal colon crypts were symmetrically bisected and the right halves of each were analyzed. The total number of positively stained cells was divided by the total number of cells in the crypt column to generate the percentage of apoptotic or proliferative cells for each column.

Statistics: Analysis for all data was determined using one-way ANOVA or a student t-test Using JMP Pro 10. Differences were considered significant if $P<0.05$.

Results

E_2 protects against TNBS induced weight loss and increases colon length: All four groups of mice had significant weight loss from their pre-study weights. WT control treated mice lost significantly more weight than WT E_2 treated mice. There was no difference in weight loss between ERβKO mice in response to E_2 (Figure 4.1A). Additionally, E_2 treatment increased the length of the colon by approximately 0.5 cm in WT but not ERβKO mice (Figure 4.1B).

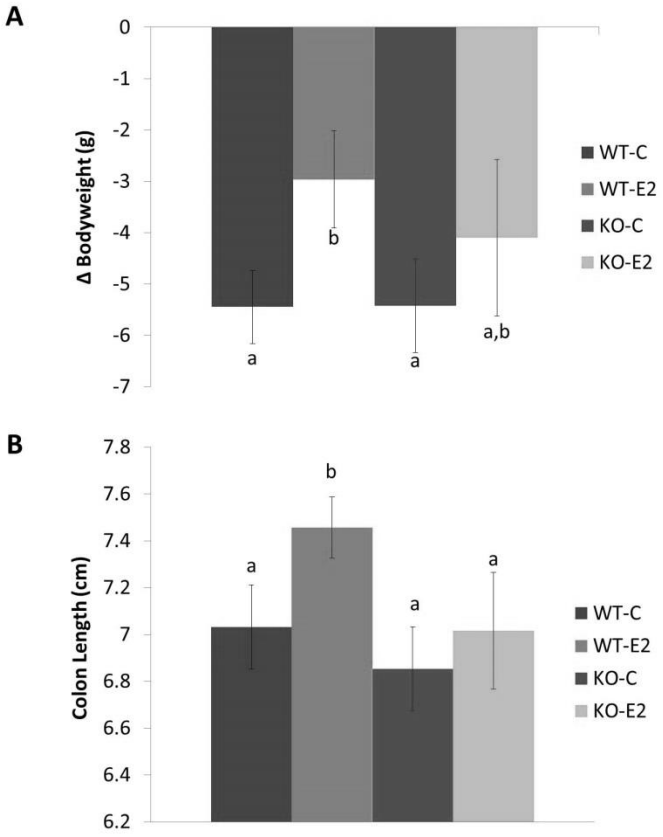

Figure 4.1. (A) Effect of E_2 on TNBS Induced Weight Loss. Values are mean weight loss per animal in grams ± SEM, and (B) Effect of E_2 on Colon Length. Values are mean colon length ± SEM. n = 12 -16 mice per group. Bars without a common letter differ, $P \leq 0.05$.

E_2 protects against inflammation and injury in the colon: After induction of acute colitis using TNBS, E_2 treated WT mice had reduced inflammation in the middle region of the colon. ERβKO mice had reduced inflammation in response to E_2 in the distal end of the colon (Figure 4.2A). There were no changes in injury scoring in the WT mice for any region of the colon. The ERβKO E_2 treated mice, however, a reduction

in injury was observed in the proximal, distal and overall colon compared to ERβKO control treated mice (Figure 4.2B).

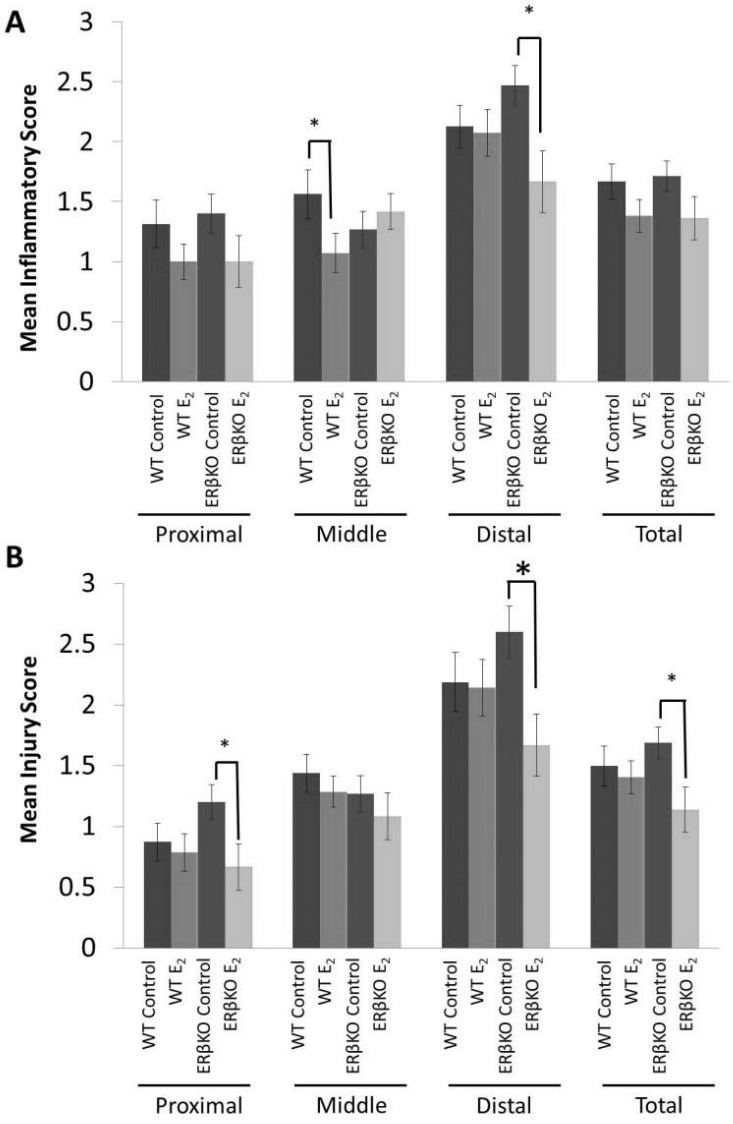

Figure 4.2. Inflammation was induced in the colon of ovariectomized mice with TNBS. Sectioned tissues were H&E stained and analyzed by a board certified pathologist. (A) Inflammation Scores and (B) Injury Scores. Values are mean score ± SEM. n = 12 - 16 mice per group. "*" denotes significance between indicated groups $P<0.05$.

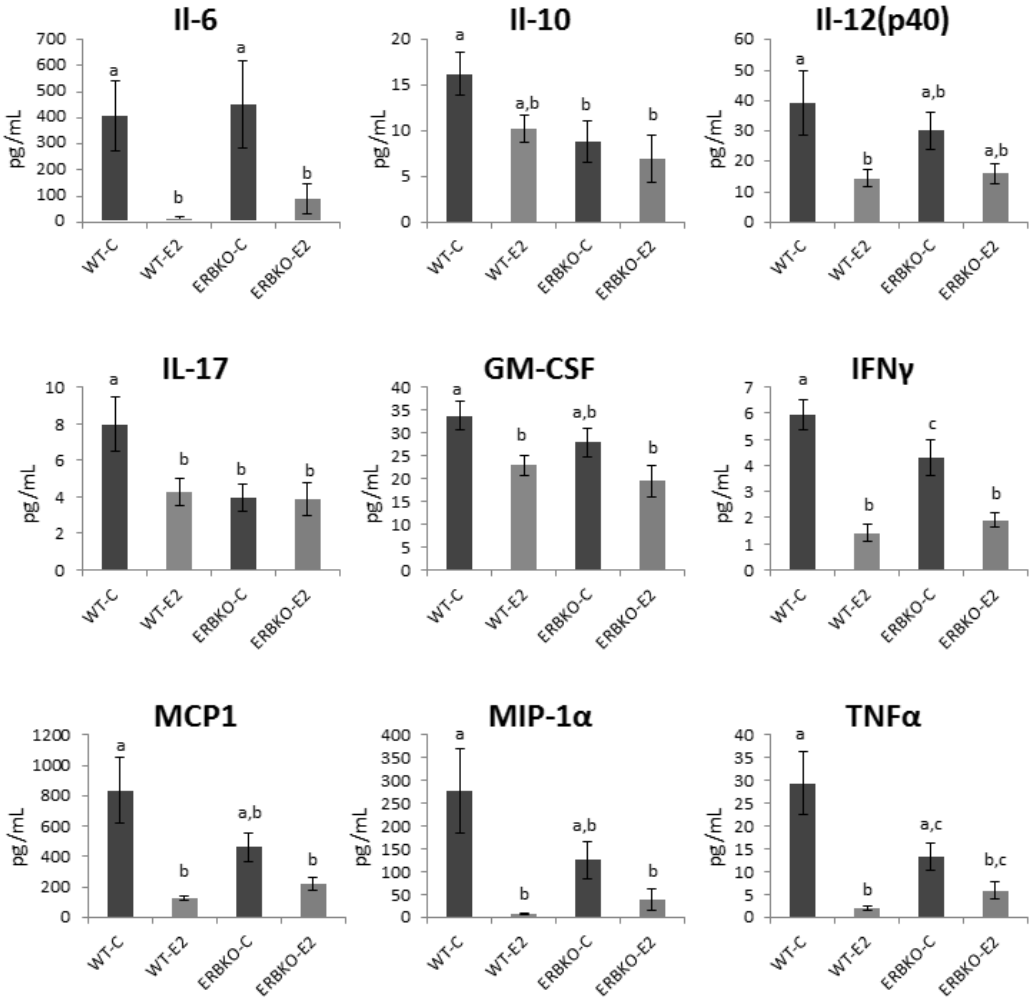

Figure 4.3. Cytokine expression in colon tissue was assessed using a multiplex assay. Values are mean expression ± SEM. Bars without a common letter differ, $P < 0.05$.

E_2 reduces the expression of pro-inflammatory cytokines: Cytokine expression profiles were determined using a Multiplex magnetic bead assay. In WT mice, treatment with E_2 resulted in reduced expression of IL-6, IL-12(p40), IL-17, GM-CSF, IFNγ, MCP-1, MIP-1α, and TNFα. In ERβKO mice, IL-6 and TNFα expression were significantly down regulated by E_2. Several other cytokines, IL-12(p40), GM-CSF,

MCP-1, MIP-1α, and TNFα, trended towards reduced expression in response to E_2 in ERβKO mice, however the differences did not reach signifigance (Figure 4.3).

Proliferation is increased in ERβKO mice: Proliferation was assessed by BrdU incorporation into actively dividing cells. Both the proximal and distal ends of the colon were analyzed. The crypts were further divided into bottom, middle and top regions. No changes in proliferation were observed in either genotype in response to E_2 in the proximal colon for any region in the crypt (Figure 4.4A). In the distal colon, E_2 treated ERβKO mice had increased proliferation compared to control treated ERβKO mice in the bottom third of crypts. No other significant differences were observed in the distal colon (Figure 4.4B).

Apoptosis is decreased in ERβKO mice: Apoptosis was analyzed in the proximal and distal colon using a TUNEL assay. Again, the crypts were divided into bottom, middle and top regions to observe changes in the different populations of colonocytes. In the proximal colon, E_2 treated ERβKO mice had reduced apoptosis both overall and in the top third of colon crypts compared to control ERβKO mice (Figure 4.5A). There was a trend for increased apoptosis in E_2 treated ERβKO mice in the distal colon compared to ERβ controls however no significant changes occurred in the distal colon in response to E_2 in any region of the crypt for either genotype (Figure 4.5B).

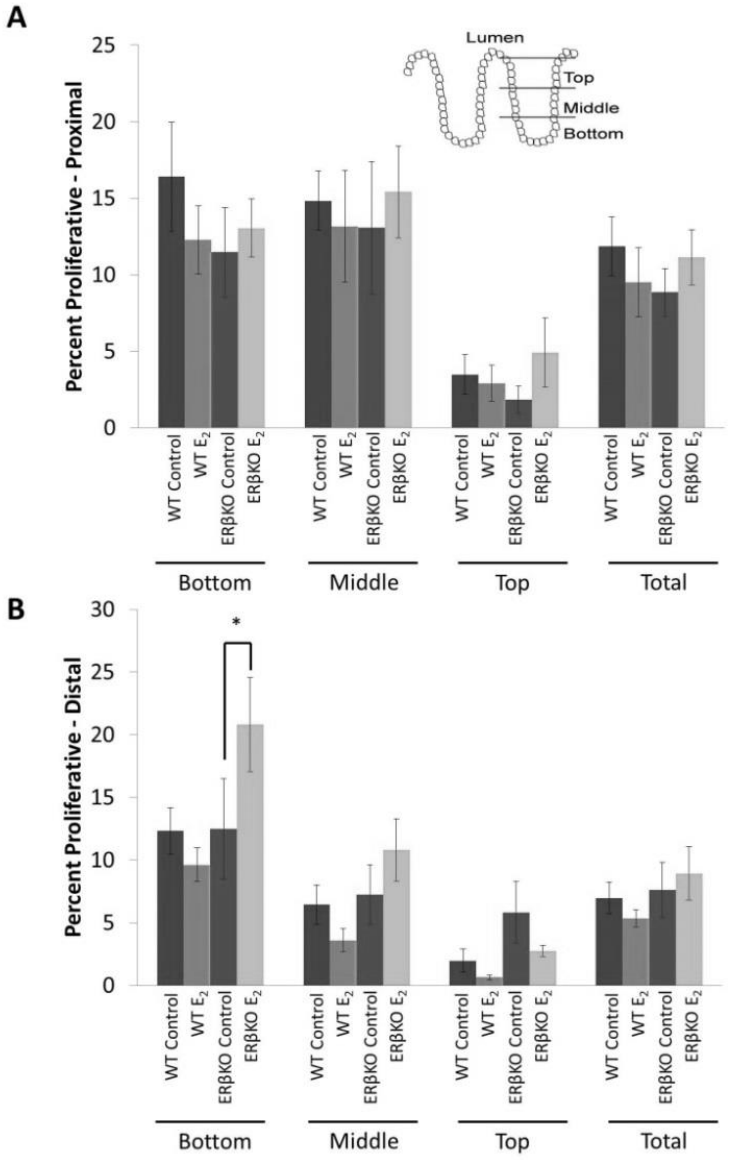

Figure 4.4. Effect of E_2 on proliferation during acute inflammation. Two hours prior to sacrifice, mice were injected with 5-bromo-2'-deoxyuridine (BrdU). Immunohistochemistry for BrdU was performed on sectioned tissues from the proximal and distal colon. Data are expressed as the percentage of proliferative cells compared to the total number of cells for either the full crypt column or the indicated region of the crypt. Data are representative of 20 well-oriented crypts per animal per proximal or distal colon with n =12 to 16 mice per group. (A) Proliferation in the proximal colon and (B) Proliferation in the distal colon. "*" denotes significance between indicated groups $P<0.05$.

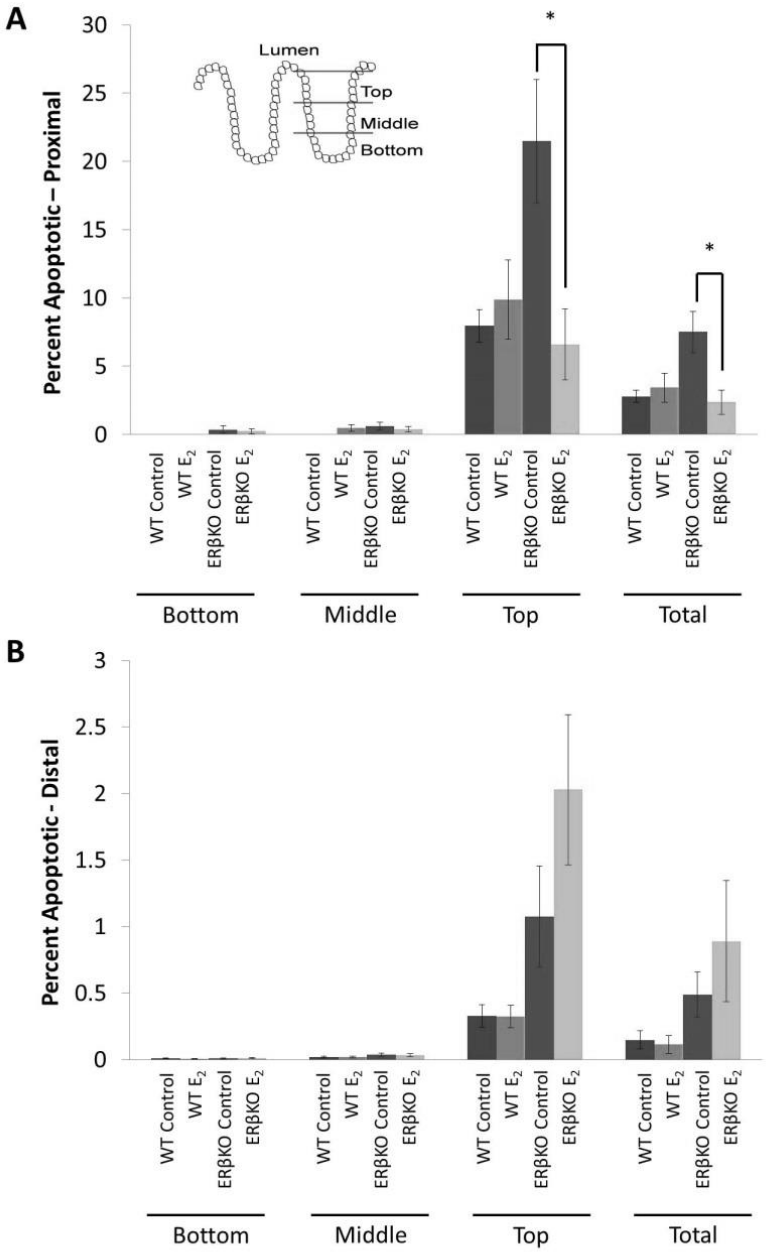

Figure 4.5. A TUNEL assay was performed on sectioned tissues from the proximal and distal colon. (A) Apoptosis in the proximal colon and (B) apoptosis in the distal colon. Data are expressed as the mean percentage of apoptotic cells compared to the total number of cells for either the full crypt column or the indicated region of the crypt ± SEM. Data are representative of 20 well-oriented crypts per animal per proximal or distal colon with n =12 to 16 mice per group. "*" denotes significance between indicated groups, $P<0.05$.

Discussion

Epidemiological studies suggest that E_2 is protective against inflammation-associated colon tumor formation [111]. One mechanism by which this protection may occur is through the modulation of acute inflammatory episodes in the colon. This idea is supported by the fact that women with Crohn's disease report that their IBD symptoms worsen during menstruation, when circulating E_2 levels are lowest [112]. Thus far, however, *in vivo* studies have been inconclusive on the effects of E_2 on inflammation and inflammation-associated colon cancer.

In this study, TNBS was used to mimic acute Crohn's disease inflammation. With this model, E_2 treatment was observed to protect against inflammation in WT and ERβKO mice in the middle and proximal regions of the colon respectively. Other studies utilizing dextran sulfate sodium (DSS) as the inflammatory reagent that models ulcerative colitis have observed that the presence of E_2 exacerbates inflammation in the colon [114]. Crohn's disease and ulcerative colitis are physiologically distinct diseases and the differences observed in response to the presence of E_2 are likely due to the inherent differences in the morphology of each disease as well as the models used to study them.

In this study, inflammation was observed to be decreased in the middle and distal colon in WT and ERβKO E_2 treated mice respectively. The likely mediator of the reduced inflammation is the decreased expression of pro-inflammatory cytokines also observed in response to E_2. Aberrant expression of cytokines is a hallmark for Crohn's disease, and several of the current treatment therapies are aimed at blocking cytokine

activity [172]. Cytokine signaling networks are complicated; involving several cell types and multiple positive and negative feedback loops. In the case of Crohn's disease, antigen presenting cells, such as dendritic cells and macrophages, secrete IL-6, IL-1, IL-12, IL-18, IL-23, and IL-27 which in turn promote T-cells to secrete more cytokines activating macrophages and inducing inflammation [172]. IL-6, IL-12(p40), IFNγ, GM-CSF, MIP-1α and, TNFα have previously been shown to be upregulated in TNBS induced colitis [173]. In WT mice in this study, the 97% reduction in IL-6 could be reducing T-cell secretion of IL-17, TNFα, and IFNγ. The lower TNFα, and IFNγ expression would result in decreased macrophage activation, further downregulating IL-6. The reduction of T-cell secreted IL-17 would in turn cause expression of IL-6, GM-CSF, MCP-1 and TNF-α to decrease.

While cytokine production was also reduced in the ERβKO mice, only expression of two of the targets I analyzed, IFNγ and IL-6, was downregulated compared to the eight targets affected in the WT mice. Studies have shown that macrophages express ERβ and that ERβ specific agonists can influence cytokine production [174, 175]. The lack of ERβ in the ERβKO mice could be reducing the ability of these cells to respond to E_2.

In immune cells, such as dendritic cells and macrophages, ERα is the predominate ER. Additionally, the spleen and thymus, which are involved in maturing immune cells, are ERα expressing tissues [176]. In both the WT and ERβKO mice in this study, E_2 could be acting through ERα. Another receptor involved could be the non-classical ER, G-protein coupled receptor 30 (GPR30). GPR30 has been observed to

reduce toll-like receptor 4 (TLR4) expression on macrophages in response to E_2 [177]. TLR4 binds lipopolysaccharide from Gram-negative bacteria and activates the innate immune system and reduction in the expression of this protein could be a method for the reduction of inflammation observed in the present study.

Not only was inflammation reduced by E_2, but injury was also mitigated. For all groups, injury scoring was highest in the distal colon, likely a result of the intrarectal method of TNBS administration. The insertion of the gavage tube into the colon physically damaged this portion of the tissue. Due to this fact, the results observed in response to E_2 in the distal colon in regards to injury are partially reflective of wound healing and not solely inflammation.

E_2 treatment was associated with a lower injury score in ERβKO mice in the proximal, distal, and overall colon. The fact that for both inflammation and injury lower scores were observed in the presence of E_2 suggests that during acute inflammation, ERβ is not the primary receptor through which E_2 elicits its effects. While ERβ is the primary ER in the colon, studies have shown that the colon also expresses ERα. As opposed to ERβ, ERα is associated with increased cell growth. It is possible that E_2 binding to ERα could be the cause of the increased proliferation in the bottom third of crypts in the distal colon of E_2 treated ERβKO mice compared to control ERβKO mice. This increase in proliferation may be the underlying mechanism behind the reduction in injury in the distal colons of these same mice. By increasing the rate of new cell growth, the ERβKO E_2 treated mice could be regenerating the tissue that was damaged during TNBS administration resulting in a less injured colon.

In the proximal colon, the reduction in apoptosis observed in E_2 treated ERβKO mice compared to control ERβKO mice is likely a downstream effect of the tissues being less damaged. In this instance, E_2 may not directly be causing the apoptosis by binding to ERs (ERα or otherwise) but rather, due to the lower levels of damaged colonocytes, the need for these cells to apoptose to repair the tissue is diminished resulting in the reduction in apoptosis. If this were the case, then the reduction in apoptosis is reflective of the protective effect of E_2 in the colon.

The lack of effect on injury in E_2 treated WT mice could be due to competitive binding for E_2 by the different ERs. If in fact the protection against injury is due to ERα, in the WT mice, the ERβ could be tying up the available E_2, preventing it from binding to ERα.

Other models of acute colonic inflammation, particularly those utilizing DSS, have shown that E_2 worsens markers of colonic inflammation. Heijmans et al. observed that WT mice receiving E_2 and induced with inflammation using DSS had a higher disease activity index, had increased weight loss and significantly increased production of the pro-inflammatory cytokine interleukin-6 (IL-6) [114]. Verdú et al also found that E_2 increased histological inflammatory scores [115]. Contrary to TNBS, DSS induces inflammation that models ulcerative colitis. DSS is believed to function via cytotoxicity resulting in loss of integrity of the mucosal barrier whereas TNBS is a haptenating reagent causing an allergic response from the host in the colon. Studies that have used DSS as the inflammatory agent have observed that E_2 treated animals do not tolerate DSS well and have a poor survival rate compared to controls [114]. This poor tolerance

is likely related to the worsening of inflammatory symptoms observed in DSS/E_2 models.

Structurally similar to E_2, phytoestrogens such as genistein are also known to bind to ERs. In other studies using TNBS to induce inflammation, high phytoestrogen diets were observed to increase acute colonic colitis. Seibel et al. observed that rats exposed pre-natally and post-natally to diets high in phytoestrogen content had increased myeloperoxidase activity, increased colon weights, and ulceration compared to controls, all indicative an increase in inflammation [178]. While phytoestrogens often have similar effect to E_2 in estrogen responsive tissues, this is not always the case. Additionally the differences observed between this study and those done with phytoestrogens could be due, in part, to changes in the treatment protocol and protocol used to induce TNBS colitis. The rats in the Seibel et al. study were exposed to the phytoestrogens from conception onward. Additionally, the rats in their study did not receive a pre-treatment of TNBS [178].

Overall, data from this study suggest that E_2 is protective against acute TNBS induced colitis and reduces the expression of pro-inflammatory cytokines in the colon. This protective effect is not mediated through ERβ and further investigation will be required to determine the molecular mechanism behind the protection observed in the presence of E_2. These data are an important step towards understanding how E_2 protects against symptoms of IBD in women with Crohn's disease and could lead to the development of treatment strategies for relieving IBD symptoms in clinical settings.

CHAPTER V

THE EFFECTS OF ESTROGEN RECEPTOR ALPHA AND ESTROGEN RECEPTOR BETA IN COLONOCYTES *IN VITRO*

Introduction

The primary estrogen receptor (ER) in the colon is ERβ. In general, this receptor is associated with decreased cell growth and induction of apoptosis. Studies form our laboratory and others have demonstrated that expression of ERβ is requisite for the protection against colon tumorgenesis in response to estradiol (E_2) the colon for both sporadic and inflammation associated models of colon cancer development as well as in human studies [63, 72, 167, 179]. As colon tumors develop, it has been observed that the expression of ERβ decreases resulting in a loss of effect in response to E_2 [87, 88, 162, 163].

The other classical ER in humans is ERα. Contrary to ERβ, ERα is associated with an increase in cell division when activated. In tissues where ERα is the predominate ER, E_2 is associated with amplified cancer cell growth. A study from our laboratory observed that in addition to the loss of ERβ expression as colon tumors developed, ERα expression was upregulated. This shift in ER expression was accompanied by a change in the physiological response to E_2 as compared to previous studies; apoptosis was downregulated while proliferation was upregulated [72].

Both ERα and ERβ are nuclear receptors primarily located within the nuclear membrane. Upon activation, they dimerize, forming either homo or heterodimers and

translocating into the nucleus where they bind to estrogen response elements located within the promoter region of target genes. Once bound, they recruit either co-activators or co-repressors to up or downregulate expression of the gene.

One protein whose expression has been demonstrated to be influenced by E_2 *in vitro* is p53. The p53 protein is a prominent tumor suppressor protein and is known to induce cell cycle arrest, apoptosis and DNA repair in response to DNA damage or other forms of cellular stress [180, 181]. Loss of heterozygosity of p53 or dominant negative mutations to the gene often occur during cancer progression. Mutations to *p53* occur in over 50% of colon tumors and loss of proper function of the protein is associated with an increase in disease severity [169, 182-185]. Studies from our laboratory have observed that E_2 increases p53 expression levels in non-malignant colonocytes and influences the mRNA expression of downstream p53 target genes [69]. In other studies where ERβ is expressed in malignant cell lines, such as some breast cancer cell lines and endometrial carcinoma cells and osteoblasts, E_2 has also been demonstrated to alter expression of p53 and its downstream targets [186-189]. This upregulation of p53 could, in part, be how E_2 initiates apoptosis of colonocytes and protects against colon tumor formation.

The present study aims to elucidate the differing role of ERα and ERβ in non-malignant colonocytes and a homologous cell line with a mutated *p53*. By better understanding how each of the ERs influence the physiology of colon cells, improved strategies for preventing colon cancer in humans may be developed.

Materials and Methods

Cells: Young adult mouse colonocyte (YAMC) bleo/neo and mp53/neo cells were generously provided by Dr. Hartmut Land (University of Rochester Medical Center). Cells were cultured in RPMI 1640 (Sigma Aldrich) with 10% fetal bovine serum (FBS; HyClone); 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences); and 1% gentamicin (GIBCO). For general maintenance, cells were placed at 33°C with 5 units of γ-interferon (IFNγ) per mL medium (Roche) on rat tail collagen type I (BD Biosciences) coated plates. Cells were cultured in media with 2x charcoal-dextran stripped FBS in place of regular FBS for 72 hr prior to receiving any experimental treatments. During experiments, cells were placed at non-permissive conditions; 39°C in the absence of IFNγ. Treatments, E_2, 4,4',4"-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT, an ERα specific agonist) and diarylpropionitrile (DPN, an ERβ specific agonist) (Tocris; Bristol UK) were prepared as 1000x stocks in ethanol (EtOH) and delivered as 1 μL/mL medium to achieve the final concentration as indicated.

Cell number assay: YAMC bleo/neo and mp53/neo cells were seeded at 15,000 cells per well on six-well plates and grown under non-permissive conditions. Cells were treated with vehicle or 1 nM E_2, 1, 5 and 10 nM PPT, and 5, 25 and 50 nM DPN as indicated for 96 hr. Forty-eight hours after the first treatment, the media was changed and treatments were replenished. At the end of the 96-hr treatment period, cells were trypsinized and collected for counting. Cell concentration was determined using a Beckman Coulter particle counter. Twenty micro-liters of sample were diluted in 10 mL

Isotone II diluent (Beckman Coulter). Samples were counted in triplicate. Three wells per treatment per experiment were used and three replicate experiments were conducted.

ERα transfected cells were counted using the same method following the transfection protocol. Treatments were again refreshed after 48 hr with cell number being assessed 96 hr post transfection.

Apoptosis: YAMC bleo/neo cells (15,000/well) were seeded on 6-well plates and grown in stripped serum medium under non-permissive conditions. Cells were treated with vehicle or 1 nM E_2, 1, 5 and 10 nM PPT, and 5, 25 and 50 nM DPN for 96 hr. Forty-eight hours after the first treatment, the media and treatments were replaced. At the end of the 96-hr treatment period, cells were trypsinized and collected. After collection, cells were centrifuged and the medium was replaced with lysis buffer from the EnzChek Caspase-3 Assay kit no. 2 (Invitrogen). The Invitrogen protocol was followed for this procedure. Fluorescence was measured on a TECAN infinite M200 plate reader. Three wells per treatment per experiment were used and three replicate experiments were conducted.

RT-PCR for p53 targets: YAMC bleo/neo and mp53/neo cells (25,000/well) were seeded in 6-well plates and maintained under non-permissive conditions in stripped serum media. Cells were grown for 72 hr and treated with 1 nM E_2 or vehicle for the final 18 hr. Cells were trypsinized and centrifuged, and RNA isolation conducted using the RNAqueous-4PCR kit (Ambion). 1 μg total RNA was used for cDNA synthesis using the Transcriptor First Strand cDNA Synthesis kit (Roche). RT-PCR samples contained 9.5 μL FastStart Universal SYBR Green Master Mix (Roche), 1.25 μL

forward and reverse primers (18s F-TCA AGA ACG AAA GTC GGA GGT T, 18s R-GGA CAT CTA AGG GCA TCA CAG, p53 F-AAA GAA AAA ACC ACT TGA TGG AGA GT, p53 R-CGG AAC ATC TCG AAG CGT TTA, Bax F-CAC CAG CTC TGA GCA GAT G, Bax R-GCG AGG CGG TGA GCA CTC C, Bcl-2 F-ATC TTC TCC TTC CAG CCT GA, Bcl-2 R-TCA GTC ATC CAC AGG GCG AT, NOXA F-GAA ATG CCT GGT ATT GGA TGG A, NOXA R-GAA CTC AT CCT ATC TCC TTC ATC AT, p27 F-GGC CAA CAG AAC AGA AGA AAA TGT, p27 R-GGG CGT CTG CTC CAC AGT or PUMA F-GCG GCG GAG ACA AGA AGA, PUMA R-GGA GTC CCA TGA AGA GAT TGT ACA; Sigma–Aldrich), 11 µL RNase-free water, and 2 µL cDNA. RT-PCR was run on a Bio-Rad iQ5 thermocycler for 45 cycles.

Transfection of ERα: Bleo/neo and mp53/neo cells were plated on 12-well plates (10,000 cells/well) in stripped-serum media for 72 hr prior to transfections. Per well, 6 µL FuGene 6 (Promega; Madison, WI), 44 µL Opti-MEM (Gibco: Grand Island, NY), and 2 µg ERα plasmid. This was incubated at room temperature for 10 min and then 2 µL CombiMag (OZ Biosciences; Marseille, France) was added and the mixture was incubated 30 more minutes at room temperature. Next, 50 µL was added to each well in 1mL media and the plate was placed on a magnet for 20 min. Following incubation on the magnet, the transfection solution was removed from the cells, fresh media was added to the cells and cells were placed at non-permissive conditions. For transfection experiments where treatments were added to the cells, treatments were included in the fresh media post-transfection.

ERα protein quantification: Protein was extracted from ERα transfected bleo/neo and mp53/neo cells using 50 μL lysis buffer for 30 min at room temperature 0, 6, 12, 18, and 24 hr post-transfection. Total protein concentrations were determined using the DC Protein Assay (Biorad; Hercules, CA) following the manufacturer's instructions. ERα protein concentration was determined by Western blot using the Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate kit (Millipore); using the methodology as previously described [190]. Antibodies were rabbit-anti-ERα (Santa Cruz Biotechnologies; Santa Cruz, CA), mouse-anti-actin (Sigma; St. Louis, MO) and goat-anti-rabbit and goat-anti-mouse IgG (Assay Designs).

Statistics: Analysis for all data was determined using one-way ANOVA using JMP Pro 10. Experiments were run in triplicate. Differences were considered significant if $P<0.05$.

Results

Functional p53 is required for DPN and E_2 to reduce cell number: Bleo/neo and mp53/neo cells were treated with E_2 and varying concentrations of PPT and DPN as indicated. Cell number was reduced in bleo/neo cells but not mp53/neo cells with E_2 and the ERβ specific agonist, DPN at all three concentrations. PPT, an ERα specific agonist, had no effect on cell number for either cell line (Figure 5.1).

DPN and E_2 induce apoptosis: Apoptosis was measured in bleo/neo cells using a caspase assay. Cells treated with both 1 nM E_2 and DPN at 50 μM displayed increased caspase-3 activity, indicating increased apoptosis for these treatment groups. PPT had no

effect on caspase activity at any concentration (Figure 5.2). Apoptosis was not assessed in mp53/neo cells due to the fact that previous studies from our laboratory have observed no change in apoptosis in response to E_2 in this cell line [69].

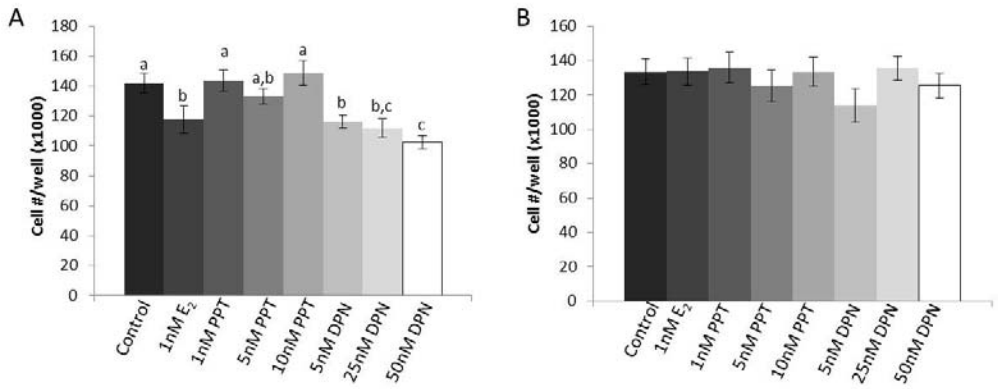

Figure 5.1. Effect of specific agonists on cell number. A cell number assay was conducted in (A) bleo/neo and (B) mp53/neo cells in response to E_2, DPN, and PPT at the concentrations indicated. Bars without a common letter differ, $P<0.05$.

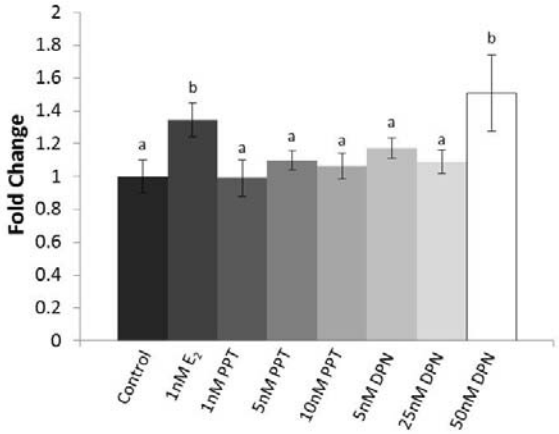

Figure 5.2. Apoptosis in response to E_2, DPN, and PPT was measured in bleo/neo cells using a caspase-3 assay. Bars without a common letter differ, $P<0.05$.

E_2, DPN and PPT all influence expression of p53 and its downstream targets: Knowing that apoptosis was induced by both E_2 and DPN in bleo/neo cells, I next wanted to see whether modulation of the p53 pathway was involved in the increased apoptosis. Expression of the pro-apoptotic downstream targets of p53, NOXA, PUMA, BAX, the anti-apoptotic Bcl-2, p27, which is a cell cycle related gene and p53 itself were measured using RT-PCR. Expression of p53 mRNA was increased by all three treatment groups. BAX expression increased 24 and 29 fold over EtOH treated controls in response to E_2 and DPN respectively. NOXA expression was induced by both E_2 and PPT, but not DPN. PPT and DPN, but not E_2 induced PUMA mRNA expression. Bcl-2 expression was downregulated by both E_2 and DPN. No treatment had an effect on p27 mRNA expression (Figure 5.3).

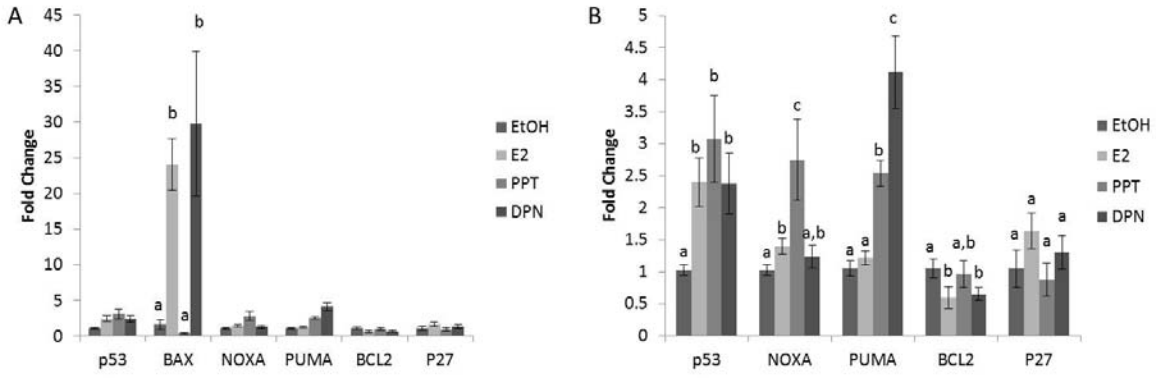

Figure 5.3. mRNA expression of p53 and several of its downstream targets was assessed using RT-PCR. (A) Depicts all targets analyzed while (B) has BAX removed from the bar graph so changes in the remaining targets are visible. Bars are fold change compared to vehicle treated controls for each gene, ± SEM. Bars without a common letter for gene target analyzed represent significant differences for that target, $P<0.05$.

ERα overexpression negates the protective effect of E_2: To mimic what was observed *in vivo* [72], ERα was overexpressed in bleo/neo and mp53/neo cells using transfection. In the bleo/neo cells, overexpression of ERα results in a loss of response to E_2. No changes in the effect of E_2 were observed in the mp53/neo cells. When ER specific agonists were administered to ERα transfected bleo/neo cells, PPT increased cell number while DPN reduced cell number (Figure 5.4).

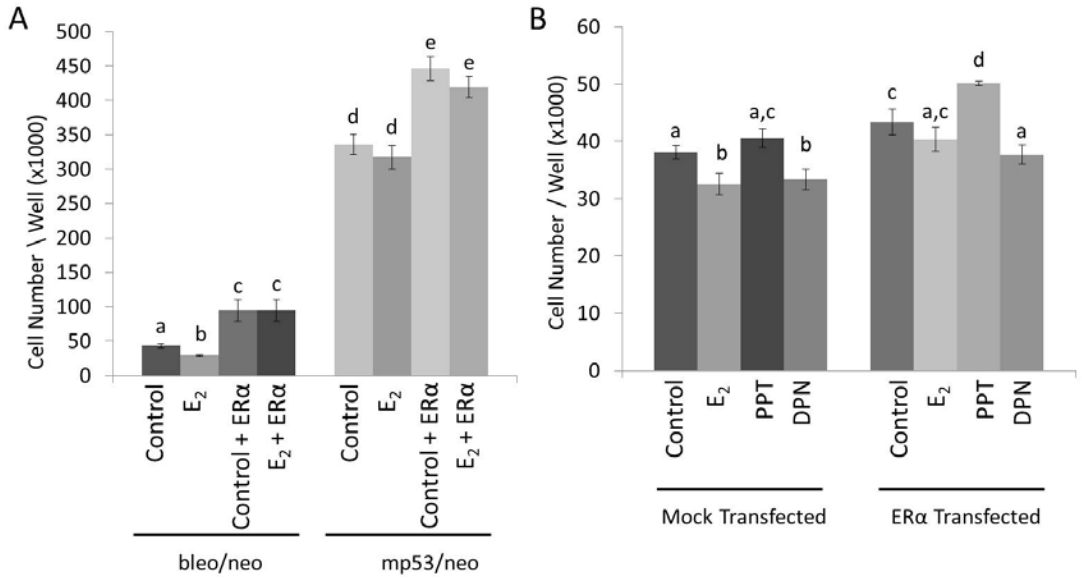

Figure 5.4. Effect of E_2 and ER specific agonists on cell number in ERα transfected cells. (A) bleo/neo and mp53 cell lines were transfected with ERα and treated with 1 nM E_2 or EtOH as a control and a cell number assay was performed. (B) ERα transfected bleo/neo cells were treated with 1 nM E_2, 10 nM PPT, or 25 nM DPN and a cell number assay was conducted. Bars are mean cell number per well of 9 replicate experiments ± SEM. Bars without a common letter defer, $P<0.05$.

Discussion

The relationship between ERα and ERβ is complex. Several tissues and systems throughout the body express both receptors, including the breast, bone, central nervous system and cardiovascular system, and the interplay between the two ERs leads to proper tissue function. Aberrant expression of one ER or the other can occur during the development of hormone-related cancers. For example, in the breast, proper ERα expression is lost in roughly 15% of human breast cancers, resulting in resistance to anti-estrogen therapies in these women [191]. Alternatively, other studies suggest that the loss of ERβ expression in breast carcinomas may be the reason behind the loss of effectiveness of anti-estrogen effectiveness at treating the disease. This loss of ERβ expression is thought to be due to hypermethylation of the promoter region of the gene [192-194].

In colon cancer, expression of ERβ has been shown to decrease as colon tumors develop both *in vivo* and in human studies [87, 88, 162, 163]. A study from our laboratory has demonstrated that in addition to the loss of ERβ, during inflammation-associated colon tumorgenesis ERα expression concomitantly increases. In the present study, I use an *in vitro* colon cell model, specific ER agonists and transfection of ERα to better understand the differing actions and physiological roles or ERα and ERβ in the colon.

When a cell number assay was performed in both non-malignant mouse colonocytes, YAMC bleo/neos, and in a homologous cell line with a mutated p53, YAMC mp53/neos, cell number was observed to decrease in the bleo/neos in response to

both E_2 and the ERβ specific agonist DPN, but not in response to the ERα specific agonist PPT. This suggests that, as had been previously proposed, ERβ is the primary ER through which E_2 exerts its protective effect in colonocytes. These same treatments in the mp53/neos had no effect, indicating that a functional p53 is requisite for the protection observed with E_2.

Related to p53, which is an important tumor-suppressor gene known to initiate cell cycle arrest, apoptosis, and DNA repair, I next measured induction of apoptosis in the bleo/neo cells. Both 1 nM E_2 and 50 nM DPN significantly induced apoptosis as measured by caspase-3 activity. Interestingly, analysis of the mRNA expression of downstream p53 targets revealed that all three agonists influenced the expression of the targets despite only E_2 and DPN resulting in quantifiable changes in apoptosis and cell number.

YAMC bleo/neo cells have been observed to express both ERα and ERβ at the mRNA and protein level [69]. Therefore, it is feasible that PPT binding to the receptor could be influencing p53 targets. The fact that no changes in cell number and apoptosis were observed in response to PPT in the bleo/neo cells suggests that perhaps the upregulated pro-apoptotic mRNA transcripts are not being translated into protein. Alternatively, the PPT could also be instigating pro-proliferative pathways that could counteract any changes in apoptosis resulting in the lack of change in cell number. Further investigation is required to understand the function of ERα as it relates to these genes in the colon.

Once I knew the roles of ERα and ERβ in the bleo/neo and mp53/neo cells with endogenous ER expression levels, I next wanted to mimic what I had observed in the inflammation-associated colon tumor development where ERα expression was increased. ERα was overexpressed in bleo/neo and mp53/neo cells using transfection. The higher levels of ERα protein in bleo/neos abrogated the protective effect of E_2. Using the specific ER agonists in the transfected cells revealed that the lack of change in cell number in these cells was due to the effects of ERα and ERβ canceling each other out; DPN reduced ERα overexpressed bleo/neo cell number while PPT increased cell number. The fact that DPN effectively reduced cell number even when ERα was overexpressed suggests that ligands specific to ERβ could be a possible treatment option for colon tumors that still express some ERβ.

In the mp53/neo cells, ERα overexpression had no effect on the lack of response to the cells to E_2 in regards to cell number. Further study is required in this cell line when transfected with ERα to observe the individual effect of ERα and ERβ specific ligands. Additionally, experiments knocking down ERβ expression will need to be conducted to further understand how loss of this ERs expression affects the physiology of colonocytes.

While additional experiments are needed to continue to explore the dynamic between ERα and ERβ as inflammation-associated colon tumors develop, the data presented in this study are critical towards understanding the roles of these receptors in colonocytes. By understanding how these receptors function in colonocytes, improved

strategies for preventing and treating colon cancer may be developed, be it through the use of ER specific agonists or other means.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Previous studies investigating the role of E_2 during colon cancer development suggest that E_2 is chemoprotective as opposed to chemotherapeutic. Malignantly transformed cell lines, in most cases, are non-responsive to treatment with E_2 [66-68]. In non-malignant colonocytes, however, E_2 treatment is associated with a decrease in cell number and increase in apoptosis via a p53 dependent mechanism [69]. The studies presented herein demonstrate the protective effect of E_2 early during both sporadic and inflammation-associated colon cancer models. Specifically, the actions of E_2 were first investigated during the initiation stage of carcinogenesis as measured by DNA adduct formation. Secondly, the effectiveness of E_2 at preventing inflammation-associated colon tumor formation was assessed when administered following the initiation of DNA damage and induction of colitis. The final *in vivo* experiment focused on E_2 and acute inflammation in the colon in the absence of carcinogen. Lastly, *in vitro* studies were conducted to explore the differing roles or ERα and ERβ in colonocytes.

In the *in vivo* model of sporadic colon carcinogenesis, E_2 was observed to induce apoptosis following the initiation of DNA damage in the colon using the colon specific carcinogen, AOM. Also in this model, dietary fish oil was equally effective at reducing DNA adduct formation in the presence and absence of E_2. E_2 itself, however, had no effect on DNA adduct formation. Data from this study suggest that induction of

apoptosis in DNA damaged colonocytes could be a primary mechanism for the protection against eventual colon tumor formation.

E_2 was also determined to be protective against inflammation-associated colon tumor development. Here, colon tumor number and size were decreased, however, further analysis of the tumors indicated that apoptosis was downregulated and proliferation upregulated. This lead to perhaps most interesting data from this experiment: a concurrent decrease in ERβ with an increase in ERα. The shift in ER expression is likely the cause for the altered response to E_2 in regards to proliferation and apoptosis and was further explored *in vitro*.

The *in vitro* experiments further expounded on the function of ERα and ERβ in colonocytes. ERβ was observed to be the receptor through which E_2 exerted its protective effect, as seen by induction of apoptosis and a decrease in cell number in response to an ERβ specific agonist, DPN. Similar to what was witnessed *in vivo*, ERα overexpression abrogated the protective effect of E_2 on reducing cell number. Additionally, *in vitro* experiments found that the protective effects of E_2 and DPN are dependent on functional p53.

Also related to the inflammation-associated colon cancer study, a separate *in vivo* study was conducted investigating the effects of E_2 on acute inflammation in the colon. In this study, E_2 protected against both inflammation and injury in the colon via a non-ERβ dependent mechanism. Apoptosis was reduced and proliferation increased in E_2 treated ERβKO mice, likely a downstream effect of the protection against injury also observed in these mice.

Collectively, these studies suggest that E_2 is protective against both sporadic and inflammation-associated colon cancer development, primarily during the early stages of carcinogenesis. Additionally, ERα and ERβ were determined to have physiologically distinct actions in the colon. In regards to ERβ, which is widely considered to be the primary ER in the colon, its expression was found to be requisite for the protection seen during inflammation-associated colon tumor formation. For acute inflammation, however, the protection conferred by the presence of E_2 was not dependent on ERβ and in fact, the majority of the protective effects were observed in mice lacking ERβ. The information learned through these studies highlight the complex actions of E_2 in the colon and provide insight into developing future preventative strategies against inflammation and colon cancer.

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